CANCER CHEMOTHERAPY REPORTS

Part 1

May/June 1974
Vol. 58, No. 3

Cancer Chemotherapy Reports

National Cancer Institute

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

LETTERS

Daunomycin (NSC-82151) and Adriamycin (NSC-123127): A Hypothesis Concerning Antitumor Activity and Cardiotoxicity [1]

Daunomycin and adriamycin (fig 1) are effective antitumor agents for various malignancies; however, both not only produce stomatitis, alopecia, and bone marrow depression, but also can cause severe and irreversible cardiac toxicity. Di Marco et al (1) have recently reported on the cytotoxic, antiviral, and antitumor activity of daunomycin, adriamycin, and seven derivatives. This report, other data in the literature, and the experimental and clinical data on the antitumor spectrum of daunomycin and adriamycin allow one to make the following tentative conclusions concerning structure-activity relationships:

a. An intact molecule is necessary for activity, ie, both the chromophore and the sugar moiety (daunosamine) are inactive.

b. An amino group on the sugar moiety is necessary and the position and spatial distance of the amino group are important.

c. Modifications may be made at the C_9 position and these modifications (eg, $COCH_3$ for daunomycin; $COCH_2OH$ for adriamycin) may influence the antitumor spectrum but not necessarily the toxicity.

d. The pKa of the molecule may be important.

[1] Daunomycin: CAS reg. No. 23541-50-6; daunorubicin (NSC-83142).

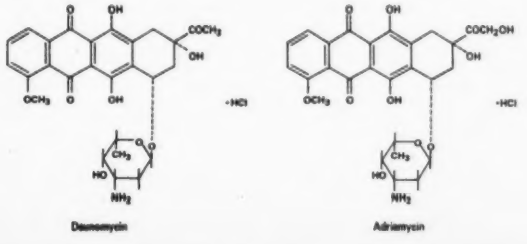

FIGURE 1.—Structures of daunomycin and adriamycin.

Based on the above considerations as well as the fact that daunomycin and adriamycin fit the N–O–O triangulation described by Zee-Cheng and Cheng (2), a common feature among various antitumor agents, I wish to propose that the cardiac toxicity in the daunomycin-adriamycin molecule resides in the daunosamine portion, allowing the molecule to be taken up by cardiac muscle while the antitumor activity generally resides in the chromophore moiety with the amino group being the only important feature contributed by the daunosamine moiety. This situation would be analogous to the digitalis glycosides, where the aglycones are more transient and less potent in their myocardial actions; the sugars attached to the aglycone are thought to enhance water solubility and cell penetrability.

If this hypothesis is correct, placement of the NH_2 group or an alkyl amino group on the chromophore at a proper spatial distance from the oxygens, for example at the C_3, C_7, or C_8 position, should give an active antitumor agent (fig 2). An additional reason for believing that the chromophore substituted with an amino group should be active is that oxytetracycline,[2] a related compound without a sugar moiety, has marginal antitumor activity against several experimental tumors.

[2] Oxytetracycline, hydrochloride: NSC-9169; CAS reg. No. 2058-46-0.

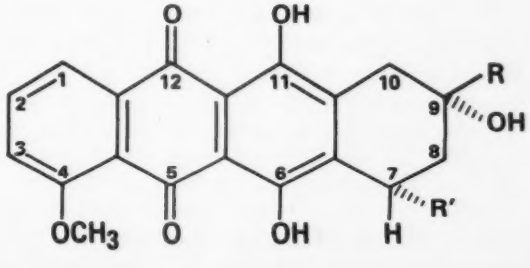

FIGURE 2.—Chromophore portion of the molecule.

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January 9, 1974

Hyperthermia in the Treatment of Cancer

The commentary "Adjuvant Temperature Effects in Cancer Therapy" by J.B. Block and C. G. Zubrod (Cancer Chemother Rep 57: 373–382, 1973) requires a reply. The authors rightly point out that although observations in the older literature on the beneficial effects of heat tend to be discarded, there is also evidence that heat can cause regression of cancer in animals and in humans. The article conveys the overall impression that the approach to the use of thermotherapy has consisted of a multitude of methods applied in poorly designed studies from which little concrete data have emerged. The further discussion of heat in combination with other modalities, such as radiotherapy and chemotherapy, serves only to cloud the issue further.

Six years ago we began to study some of the basic factors involved in the application of heat to cancer cells using animal model systems and cells in culture. This work with tumors (V × 2 carcinoma in the rabbit, Yoshida sarcoma, and mammary carcinoma in the rat) has involved in vitro-in vivo studies in which the response of the cancer cells to heat in vitro has been evaluated in relation to the effects of hyperthermia on the tumor in the host. We define hyperthermia in terms of the intratumor temperature that is measured by direct readout from needle thermistors inserted into the tumor. This gives more meaningful results than reference to the applied temperature—the temperature differential between a limb tumor and the immersion bath or perfusion fluid may be as much as 3–4 C, for example. The intratumor temperature has not been measured in much of the work reported in the older literature, and as pointed out by Block and Zubrod, various results have been obtained under different heating conditions. Our findings can be summarized as follows:

a. Hyperthermia (42 C) has a selective destructive effect on cancer cells in the systems examined, the normal tissues of the rabbit and rat being undamaged at 42 C (1–3). This confirms the effects of heat reported by previous investigators for other types of cancer cells (4–6).

b. The lethal temperature range begins at 41.5 C, and for cancer therapy the operational range for animals and for man is 41.5–42.0 C (1–3,7). Although more rapid destructive effects are achieved at intratumor temperatures in excess of 42.0 C, as previously reported by others (8,9), host tolerance considerations preclude the use of these. Above 37.5 C there is a stimulatory temperature range (37.5–40.0 C) over which the metabolism of the tumor cells is progressively increased, until at 40 C it may be double or more the control value (2,3,7). Tumors, which are cured in vivo at 42 C, grow and spread more rapidly when heated at 40 C than at body temperature.[1] Similarly, heat susceptible tumors, if heated inadequately at 42 C, exhibit enhanced spread and metastasis (10).

c. Multiplying and nonmultiplying cells are sensitive to heat, but nonmultiplying cells require longer to destroy. Cells that are not destroyed by heat recover rapidly and completely (7). Recovery of cancer cells following hyperthermia has been confirmed recently by Palzer and Heidelberger (11) who postulate that hyperthermic cell killing is at least a two-step process involving the conversion of potentially lethal damage into actual cell killing.

d. The importance of adequate heating for

[1] Dickson JA, and Ellis HA. Studies on Yoshida tumour growth in rats: the stimulatory temperature range. In preparation.

destruction of the tumor cell population (points *b* and *c*) is illustrated by the response of Yoshida tumors on the feet of rats. An intratumor temperature of 42 C maintained for 1 hour cures tumors of less than 1.5 ml in volume. At tumor volumes in excess of 1.5 ml, maintenance for 1 hour at 42 C leads to temporary restraint followed by resumed exponential growth. A 3.0-ml tumor can be destroyed by 42 C applied for 2 hours or by two sessions of 1 hour at 42 C on consecutive days.

e. Fractionated heating of tumors has advantages if the tumor cell generation time can be assessed (2).

f. The action of heat on cancer cells is specific and characteristic and differs from the action of other anticancer agents, eg, cytotoxic drugs, radiotherapy (1–3). With hyperthermia applied to the tumor (local hyperthermia) the slow absorption of dead cells leads to potentiation of the immune response with destruction of metastases. The V × 2 tumor can also be destroyed by radiotherapy, but the animal still dies from preexisting metastases (12).

g. The sensitivity of a tumor in the host animal can be predicted by in vitro biochemical tests, the most suitable of which appears to be the measurement of respiration (1–3,7). From in vitro studies, Mondovi et al previously postulated that irreversible damage to cancer cell respiration at 42 C should be regarded as a general phenomenon (4). Inhibition of metabolism at 42 C in vitro has been demonstrated for a variety of human solid tumors (5,6,13).

h. Metastases may have a different sensitivity to heat than the primary tumor (2).

These results shed light on the apparent inconsistencies in the literature regarding the action of heat on tumors. They also provide evidence for the suggestion of Block and Zubrod that heat may act to stimulate tumor growth at one temperature yet inhibit growth at another, and that the duration of the induced temperature change is important. The statement of Block and Zubrod that ". . . temperatures greater than 41 C . . . probably do not apply to potential clinical use" is not consonant with the data available on the effects of heat on cancer cells. Intratumor temperatures in the region of 42 C are feasible in humans and will be required if thermotherapy is to become a practical proposition with heating times which are realistic in terms of patient tolerance and staff man hours.

Guided by our results in animal systems, we have begun treating selected patients who have metastatic disease with total-body hyperthermia. We are using the technique of Henderson and Pettigrew (14), which employs inhalation of warmed anesthetic gases to elevate the body temperature and encasement of the patient in heated wax to minimize heat loss. This method gives safe, reproducible, and easily controlled elevation of body temperature. It can be estimated that at 42 C a heating time in the region of 20 hours will probably be required for cancer therapy in humans using total-body hyperthermia (15). There is some evidence that, as with cytotoxic drugs and radiotherapy, knowledge of the cell kinetics of the tumor population could be used for most efficient tumor destruction by elevated temperature. But cell kinetic data for human tumors are technically difficult to obtain, and we have achieved little success in this respect using biopsy material in vitro. In view of these considerations and the data already outlined for animal tumors, our current belief is that to achieve maximum benefit in human cancers we should override cell kinetic considerations and use a prolonged heating period that will kill cells irrespective of their position in the cell cycle. A heating period in the region of 10–20 hours at 42 C would seem a reasonable goal. Cavaliere et al (6) have indicated the potential of sustained elevation of tumor temperature in accessible tumors of the limbs. The older literature indicates clearly that for tumor regression following pyrexia (spontaneous from erysipelas or induced by toxins) prolonged elevation of temperature was required.

Heat is a physical entity and should therefore be capable of destroying all cancer cells. The selective susceptibility of cancer cell metabolism to heat may be the Achilles' heel ther-

apists have been seeking for many years. Maintaining the body temperature of even selected patients (ie, patients who do not have cardiovascular, respiratory, or other diseases besides cancer) in the region of 42 C for prolonged periods of time presents many problems, not only from the patient's point of view, but also from the point of view of staff care. An investigation of the effects of elevated temperature on physiologic processes, which we know little about, must constitute part of our thermotherapy program. However, until such an approach is tested, the potential of controlled hyperthermia for the treatment of metastatic cancer must remain unknown. It is important to point out in relation to Block and Zubrod's article that animals make poor model systems for total-body hyperthermia investigations. The temperature regulatory mechanisms of animals differ from those in man, and the common laboratory animals (mice, rats, rabbits) do not readily tolerate body temperatures in the region of 42 C (2,3,10).

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January 25, 1974

Hyperthermia and Cancerous Tissue Water Structure

In their apt discussion of adjuvant temperature effects in cancer therapy, Block and Zubrod (1) conclude that well-designed clinical efforts to unequivocally evaluate the effects of hyperthermia on cancer are critically needed. We suggest that further understanding can be gained from these important clinical evaluations by obtaining data on the differences in malignant and nonmalignant tissue water structure. Recent diagnostic achievements using nuclear magnetic resonance (NMR) and microwave techniques indicate that this type of information is significant.

Research on liquids and review (2) of other research on water under inanimate conditions such as capillaries, small droplets, thin films, gels, ionic solution, sands, clays, coal, etc, found the water to be highly interfa-

cially structured and anomalous in behavior. The physiochemical properties of these systems are explained by assuming some type structure (order) within the liquid extending from the interface.

Thermal anomalies were found near 15, 30, 45, and 60 C by Drost-Hansen (3) who began his studies with a careful analysis of anomalous temperature points of water and aqueous solutions. These anomalies, which appear as slope changes in the parameters, were found to be more prevalent in the case of aqueous surface and interfacial phenomena. In surface and interfacial phenomena the anomalous temperatures may shift slightly from the bulk values, with the shift for interfacial systems usually toward higher temperatures. Drost-Hansen's analysis of biologic systems revealed the same thermal anomalies and found the biologic system changes occurring at or near temperatures at which anomalies are found in nonliving aqueous solutions.

Living tissue is also highly interfacial, exhibiting biowater structure with both ordered and less ordered portions (3–10). For instance, glycoproteins have been identified as the biowater structuring agents that prevent antarctic fishes from freezing at –1.8 C (11–13). The upper lethal limit for antarctic fishes is approximately 6 C at which temperature their central nervous system tissue disintegrates (11). Based on the phase changes at the thermal anomalies it is possible to determine limits for minimum, optimum, and lethal temperatures for growth. For some forms of life there are several anomalies resulting in several growth optima. The changes in biologic systems at the thermal anomalies appear to be changes in water structure, system conformation, and/or membrane properties. The 45 C anomaly marks the approximate upper lethal temperature limit for all animate life and the 30 C anomaly marks the transition in humans between consciousness and unconsciousness. The latter anomaly is useful in certain surgical techniques.

In an extensive analysis of tissue biowater, Olmstead (14) found that both embryonic and malignant tissues have a higher biowater content than normal adult tissues and that their chemical activities differ. An increase in biowater content was found to occur as the tissue changed from normal to malignant.

That the biowater structure (relative fraction of order-disorder) is related to cancer and can be used to determine whether tissue is malignant or nonmalignant now seems accepted (8,10,15). Using NMR to show the amount of biowater ordering, Hazlewood et al (10) have extended this ability to determine the degree of malignancy: the more cancerous tissue shows less structuring.

Scientific evidence certainly indicates that at least some malignancies may be successfully treated by raising the temperature of the tumor to between 40 and 45 C for a period of time. It is believed the major mechanisms involved are: the higher water content of certain cancerous tissue, the lessened structure of the water in this tissue, and a second-order phase transition induced by the hyperthermia. The possible final result of the increased thermal energy might be the rupture of the already less structured cell wall which allows its subsequent destruction by the host's natural defenses. The initial cell destruction by thermal techniques could possibly be aided by chemical and immunologic means and the host could be afforded the already available organism assists of surgery, dialysis, and other means to reduce the toxicity of the process and the necrotized tissue.

The diagnostic success achieved using NMR (8,10) and microwaves (15) to detect malignancy by measuring water structural differences between normal and cancerous tissue is further evidence that well-designed clinical efforts to evaluate the effect of hyperthermia on cancer, as called for by Block and Zubrod (1), would prove worthwhile.

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February 5, 1974

Proposed New Derivative of Cytosine Arabinoside (NSC-63878) to Circumvent Resistance [1]

Cytosine arabinoside (fig 1) has proved useful against certain types of malignant neoplasms and is used extensively in the clinic. Its ultimate potential, however, is limited by two phenomena. First, it is rapidly deaminated by cytidine deaminase of normal host tissue to uracil arabinoside which is devoid of antitumor activity. Thus, frequent or continuous therapy is required to maintain effective blood levels. Recently, tumor cells have been shown to exhibit increased cytidine deaminase activity following cytosine arabinoside therapy, suggesting a possible resistance mechanism involving rapid metabolism. Secondly, resistance to cytosine arabinoside rapidly develops by deletion of deoxycytidine kinase, the enzyme responsible for conversion of cytosine arabinoside to its active phosphorylated form. Resistance in L1210 cells in vivo appears after only one schedule of therapy.

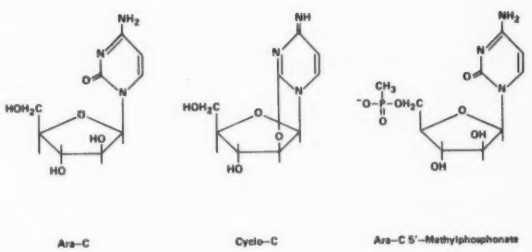

FIGURE 1.—Chemical structures of cytosine arabinoside (Ara-C), anhydro-ara-C (cyclo-C), and proposed ara-C-5'-methylphosphonate.

Numerous modifications of the basic cytosine arabinoside molecule have been made in attempts to overcome these two limitations. It now appears that the first limitation (ie, rapid deamination) has been successfully eliminated with the analog anhydro-ara-C[2] (fig 1). Ho demonstrated that anhydro-ara-C is not deaminated by human liver or mouse kidney, two tissues very high in cytidine deaminase activity (1). Venditti et al (2) reported that comparable antileukemic activity against L1210 could be obtained with anhydro-ara-C given on a daily basis, whereas cytosine arabinoside required frequent administration (q3h). Unfortunately, anhydro-ara-C does not resolve the second limitation, ie, resistance due to deletion of deoxycytidine kinase. As pointed out

[1] Cytosine arabinoside: CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

[2] Anhydro-ara-C: NSC-129220; CAS reg. No. 10212-28-9; 6*H*-furo[2′,3′:4,5]oxazolo[3,2-*a*]pyrimidine-2-methanol, 2,3,3*a*,9*a*-tetrahydro-3-hydroxy-6-imino-, monoacetate (salt); cyclocytidine.

by Venditti et al, L1210 cells resistant to cytosine arabinoside were cross-resistant to anhydro-ara-C (2). Also, the results of Ho (1) indicate that anhydro-ara-C must be converted to cytosine arabinoside before subsequent phosphorylation can occur. The difficulty in surpassing this form of resistance is in constructing an analog of the product of the deleted reaction (ara-CMP) which can pass through the cell membrane and yet still retains selective antitumor specificity. Also, the analog must retain an adequate structural similarity so that it can still function as a substrate for subsequent vital anabolic reactions (successive phosphorylation and interaction with DNA polymerase). Thus far, no analog of cytosine arabinoside has fulfilled these criteria. Based on a recent report by Wigler et al (3), synthesis of a new analog of cytosine arabinoside which could fulfill these requirements might now be possible. The structure I am proposing is ara-C-5′-methylphosphonate (fig 1). Wigler et al (3) have been working with analogs of 5-bromodeoxyuridine and have found that methylation of the 5′-phosphate of 5-bromodeoxyuridine 5′-monophosphate (not to be confused with O-methyl esters) resulted in the formation of 5-bromodeoxyuridine 5′-methylphosphonate which was cytotoxic, passed freely through cell membranes, was resistant to phosphatase attack, and was apparently incorporated into DNA (as determined by DNA sedimentation). Ara-C-5′-methylphosphonate might possess all of these properties as well as being resistant to cytidine deaminase since pyrimidine nucleotides are not substrates for this enzyme.

Therefore, it is proposed that this compound be synthesized as an extension of the rational design of cytosine arabinoside analogs and be tested for antitumor activity in the appropriate animal tumor systems.

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February 21, 1974

Pulmonary Fibrosis Following Multiple Treatment With Bleomycin (NSC-125066) in Dogs[1,2,3,4]

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SUMMARY

Bleomycin, an antibiotic with marked antitumor activity, was injected intravenously into 11 beagle dogs in doses of 0.625 mg/kg every fourth day. Seven dogs received 31–46 injections (treatment for 3½–4 months) and four dogs received 94–104 injections (treatment for 12½–14 months). All dogs developed dose-related histopathologic evidence of interstitial pneumonia. Lung lesions covered a maximum of 93% of the lung surface and a maximum of 30%–40% of the cross-sectional area. Radiographic signs for lung lesions included pericardiac opacities sometimes obscuring the heart contour, diffuse infiltration of the lung fields, and adhesions at the costophrenic sinuses. Radiographic changes were first noted 3 months after the beginning of treatment and affected all dogs that were treated for more than 8 months. Lung volumes at a pressure of 25 cm H_2O decreased to values as low as 40%–60% of control. Changes appeared after 2½ months and affected all dogs treated for 14 months. In one dog injected for 6 months, the decrease in lung compliance showed some regression after termination of treatment. Other toxic effects included skin lesions (ulcerations of paw pads, onychoptosis, ulcers at pressure points, or focal alopecia), interstitial nephritis, weight loss, and intermittent mild lymphopenia. The occurrence and severity of skin lesions did not correlate with pulmonary toxic effects.

[Cancer Chemother Rep 58:301–310, 1974]

[1] Received May 30, 1973; revised Nov 7, 1973; accepted Nov 27, 1973.

[2] Supported by contract NIH-70-2055 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health (NIH), Department of Health, Education, and Welfare.

[3] Bleomycin consists of 13 polypeptides: A_1–A_6, A'_2, and B_1–B_6. The bleomycin used in this study was free of copper and contained A_2 (50%–65%), B_2 (15%–50%), A_1 and A'_2 (20%–30%), B_4 (3%), and trace amounts of the other components (1). The following formulations of bleomycin were used: Nippon Kayaku Company, Ltd., Tokyo, Japan, sample MDF7; Bristol Laboratories, Syracuse, NY, samples MEF24 and BRS 70L622.

[4] This work summarizes data presented in the following reports: Thompson GR, Schaeppi U, Fleischman RW, et al. Bleomycin (NSC-125066) toxicity of iv injection in dogs. US Dept of Commerce National Technical Information Service, 1970, PB-194 287. Schaeppi U, Phelan RS, Stadnicki SW, et al. Bleomycin (NSC-125066): changes in lung compliance following repeated iv injections with daily doses of 0.625 mg/kg q 4 day in dogs. Informal Report 11b to Laboratory of Toxicology, Experimental Therapeutics (EX), DCT, NCI, NIH, Aug 8, 1971.

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[8] We thank our project officers, Dr. David A. Cooney and Mrs. Ruth D. Davis, Laboratory of Toxicology, EX, DCT, NCI, NIH, for advice and guidance during the performance of this work; Mr. Italo Pieri, MS, for valuable assistance with the lung compliance testing; and Dr. Harris Rosenkrantz, Mason Research Institute, for the performance of hematologic and blood chemical investigations.

The antibiotic bleomycin that is isolated from *Streptomyces verticillus* (1) has potent antineoplastic activity against experimental tumors in rodents (2–6) and activity in man against squamous-cell carcinomas (6–10), malignant lymphomas (10–13), and malignant testicular tumors (10,14). In clinical trials in man, bleomycin, in contrast with other antitumor agents, did not produce important toxic effects upon bone marrow, lymphoid tissues, and liver, but did produce toxic effects including interstitial pneumonia and pulmonary fibrosis (7,10,12,13,15,16).[9] Toxic effects of subacute treatment in dogs and monkeys included interstitial pneumonia, renal and dermal lesions, and general emaciation (17–19). Hypocellularity of the bone marrow and immunosuppressive effects were not observed (18–20).

The present study describes pulmonary toxic reactions in dogs resulting from intensive treatment with bleomycin.

METHODS

Experiments were performed on 11 beagle dogs (7–10 kg body weight) in accordance with the procedures for preclinical toxicologic evaluation of cancer chemotherapeutic agents (21,22). Physical examination, hematologic and blood chemical tests, and gross and histopathologic evaluations were performed in the same manner as previously reported. Toxicity was rated semiquantitatively (23). Eleven dogs received repeated intravenous (iv) injections of bleomycin, 0.625 mg/kg every fourth day. Four dogs were treated for 12½–14 months (94–108 injections) and were necropsied immediately after their last injection. The other seven dogs were treated for 4–6 months (31–46 injections) and survivors were kept for a posttreatment observation period of 4½–6 months.

Chest radiographs (50–54 kilovolt [peak], 25 ma, 0.05 seconds) were taken for all but one dog (No. 684 F). They were performed under barbiturate anesthesia at 4-week intervals.

The deflation static volume-pressure relationship of the lung and chest wall was investigated biweekly in five dogs treated with 32–46 doses. Lung and chest wall compliance tests were occasionally performed in three additional dogs treated with 96–108 doses. While under barbiturate anesthesia (pentobarbital sodium or thiamylal sodium), the dogs were intubated with a cuffed endotracheal tubing (Rüsch), 10 mm outside diameter, and compliance was tested in the prone position. At the end of expiration, the animals were connected to a 2000-ml acrylic supersyringe and ventilated ten times with pure oxygen. The lung was then inflated with a volume of oxygen that resulted in an endotracheal pressure of 25 cm H_2O. Oxygen was thereafter stepwise withdrawn at 15-second intervals and the volumes required to sustain transthoracic pressure of 20, 15, 10, or 5 cm H_2O were recorded. This sequence was performed six times such that each value in table 1 represents the mean of six individual determinations. Our pretest values agreed with determinations reported by other investigators (24). Figure 1 shows pressure isopleths of 25, 20, 15, 10, and 5 cm H_2O respectively, in a plot of lung volume versus time.

RESULTS

Following intensive bleomycin treatment pulmonary changes in dogs included decreased deflation volume-pressure curves, radiographic opacities, and interstitial pneumonia.

Two out of five dogs treated with bleomycin for 4½–6 months and tested biweekly had a decrease in expiratory static lung and chest wall compliance (fig 1 and table 1). In both dogs compliance began to decrease 2½ months after onset of treatment and the decrease progressed throughout treatment. In one dog (No. 684 F) on Day 127, the lung volume at 25 cm H_2O had decreased to approximately 50% of the pretreatment values. At this time, the dog died following barbiturate anesthesia. The treatment of the other dog (No. 685 M) was terminated after 6 months (46 treatments)

[9] Tsubaki S, Suzuki H, and Ogawa T. Clinical results of bleomycin in treatment of malignant tumors of the head and neck. Translation of a paper presented at the Japanese Society of Otorhinolaryngology, 69th General Assembly, Tokyo, Japan, 1968.

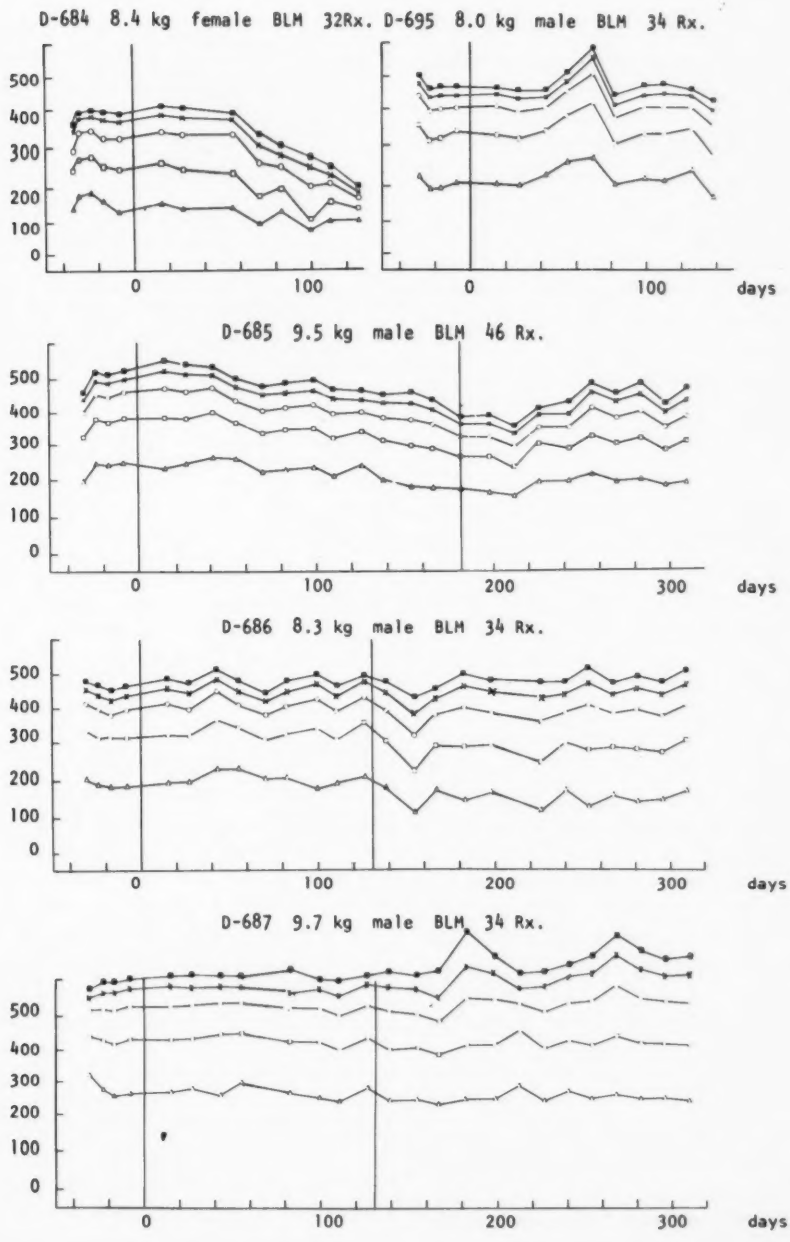

FIGURE 1.—Pressure isopleths of volume versus time in anesthetized beagles before, during, and after iv injection with bleomycin (BLM) (0.625 mg/kg every fourth day). In each record pressure isopleths represent (from top to bottom) lung volumes needed to sustain transthoracic lung pressure at a pressure of 25, 20, 15, 10, or 5 cm of water. Ordinate = lung volumes in ml, abscissa = days after onset of treatment. Vertical lines for each record = onset of treatment at left and termination of treatment at right. Notice bleomycin-induced decrease in static lung compliance in dog No. 684 and No. 685.

because of a progressive decrease in lung volumes. After termination of treatment, pressure isopleths partially returned to pretreatment values, but the dog developed a progressive azotemia and was killed for necropsy in poor condition on Day 309. Three dogs showed no changes in lung compliance throughout the treatment (4½ months) and observation (6 months) periods. The values from a deflation volume-pressure curve of one untreated control dog remained reproducible throughout a 12-month testing period.

The three dogs treated for 13–14 months and tested for compliance prior to termination of treatment showed a 40%–60% decrease in lung volumes compared with untreated dogs (table 1).

Pulmonary radiographs revealed changes in seven out of ten dogs radiographed, ie, in four of four dogs treated for 12½–14 months and in three of six dogs treated for 4–6 months (table 2). Lesions appeared 3–10 months after the onset of treatment (table 3). Changes included pericardiac opacities that sometimes obscured the heart contours (three of seven dogs) (fig 2); diffuse infiltration of apical (six of seven dogs), intermediate (five of seven dogs), and diaphragmatic (two of seven dogs) lung fields; and adhesions of the costophrenic sinus (one of seven dogs) (table 2).

All 11 dogs treated for 4–14 months developed pulmonary fibrosis. The lesions primarily affected the pleural surface and appeared as irregular, focal or confluent, slightly elevated, and firm areas of grey-tan-yellow mottling. They occurred predominantly at friction sites, such as lung margins, as described in our earlier reports (17). Pleural thickening affected as much as 93% of the pleural surface of both lungs (table 1) and interstitial pneumonia involved as much as 30%–40% of the cross-sectional area. In contrast with the lesions observed in dogs treated for shorter periods (17), more prolonged treatment resulted in more extensive parenchymal lesions. The four dogs that were kept for a posttreatment observation period of 4–6 months also exhibited white fibrotic scars in the pleura that were either well demarcated or diffuse in distribution. Similar pleural fibrosis was observed in one of the seven dogs

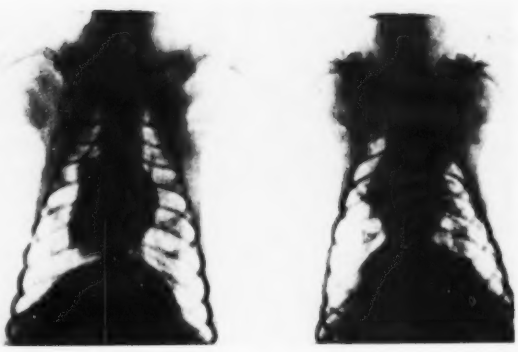

FIGURE 2.—Dog No. 624 F. Pulmonary radiograph before and during treatment with bleomycin. Left = chest radiograph before treatment (Day -3) and right = chest radiograph during treatment (Day 111, 0.625 mg/kg every fourth day, 28 doses, total dose = 17.5 mg/kg). Notice the poor definition of heart contours and infiltration of the left diaphragmatic lung field. The dog was moribund on Day 123 and the necropsy revealed lung lesions that affected 82% of the entire lung surface.

(No. 695 M) necropsied after termination of treatment on Day 140. A microscopic evaluation of the mottled areas revealed an interstitial pneumonia as described in our earlier reports (17–18), whereas the white areas consisted primarily of fibrous tissue.

Two of the 11 dogs died, and four were killed in poor general condition from treatment-related toxicity (table 2). Dog No. 624 F died unexpectedly after a treatment period of 4 months; the other dog (No. 673 M) had a progressive dyspnea and rales and died after being treated for 12½ months. In both dogs the necropsy and histopathologic evaluation revealed acute bronchopneumonia that was superimposed on interstitial pneumonia. Focal areas of acute pneumonia were also observed in two additional dogs who survived 96 and 108 treatments respectively and who were necropsied immediately after the last treatment. None of these dogs exhibited preterminal pyrexia.

Four additional dogs died following barbiturate anesthesia which was administered to perform lung compliance tests or chest radiographs. The cause of death was probably related to chronic lung disease with hypoven-

TABLE 1.—Bleomycin (0.625 mg/kg iv every 4th day in dogs): effects upon expiratory static lung compliance and correlation with lung pathology*

Dog No. and sex†	No. of treatments	Condition at autopsy	Day animal died or was killed	Lung compliance: Day of maximum change	Lung compliance: Lung volumes (ml) at H_2O pressure indicated‡ 25 cm	20 cm	15 cm	10 cm	5 cm	Gross and histopathologic evaluations: Left lung, pleural: % of surface affected — Apical	Cardiac	Diaphragmatic	Right lung, pleural: % of surface affected — Apical	Cardiac	Diaphragmatic	Azygous	Both lungs: % of surface affected
681 M	108	Poor	431	410¶	218	203	177	131	79	100	90	85	95	100	90	90	93
678 F	103	Poor§	411	410¶	241	219	189	140	90	90	90	75	75	90	80	75	83
666 F	96	Poor§	384	370¶	180	164	144	106	63	ND	ND	ND	ND	ND	ND	ND	80
685 M	46	Poor	309	208¶	253	328	291	234	150	75	55	25	75	55	25	20	47
686 F	84	Good	314	NC	NC	NC	NC	NC	NC	15	15	1	20	6	1	18	11
687 M	84	Good	314	NC	NC	NC	NC	NC	NC	30	50	8	30	15	5	5	20
695 M	84	Good§	140	NC	NC	NC	NC	NC	NC	ND	ND	ND	ND	ND	ND	ND	20
684 F	82	Good§	127	124¶	203	188	170	142	105	ND	ND	ND	ND	ND	ND	ND	60
624 F	81	Died	123	ND	ND	ND	ND	ND	ND	100	100	50	100	75	75	75	82
625 M	81	Good	258	ND	ND	ND	ND	ND	ND	70	80	85	80	75	65	45	71

* ND = not determined; NC = no change throughout entire study, *see* fig 1.

† Dog No. 673 M was not listed because of incomplete data.

‡

H_2O pressure (cm)	Mean control (6 dogs; weight range, 7.7–8.9 kg)	Standard deviation
25	465	52.2
20	436	48.0
15	395	49.1
10	310	34.4
5	188	21.4

§ Incidental death was related to barbiturate anesthesia.

¶ Tested at the end of study only.

TABLE 2.—Toxicity following iv bleomycin injection (0.625 mg/kg every 4th day) in dogs*

Dog No. and sex	681 M	678 F	666 M	673 M	685 M	686 F	687 M	695 M	684 F	624 F	625 M
No. of treatments	108	103	96	94	46	34	34	34	32	31	31
Total dose (mg/kg)	59	64	60	59	29	21	21	21	20	19	19
Day animal died or was killed	431	411	384	374	309	314	314	140	127	123	258
Condition at autopsy	Poor	Poor†	Poor†	Died	Poor			†	†	Died	
Initial body weight (kg)	7.9	7.6	7.3	7.8	9.5	8.3	9.7	8.9	8.2	7.2	9.2
Clinical condition											
Weight loss (%)	14	21	34	16	18	9	5	10		14	
Dehydration	(+)	++	++	+						+	
Integumentary lesions	++	++	++	++	+		+	+	+	++	(+)
Dyspnea				+							
Lung compliance (↓)	+++	NT	+++	+++	+				++	NT	NT
Radiographic pulmonary lesions	+	+	+++	+++	++		+		NT	+++	
Blood determinations‡											
Lymphocytes (cells/mm³)	756	662	846	1100	1284	979			826	1650	
Blood urea nitrogen (mg/100 ml)		38.4		54.4	82.6						
Sulfobromophthalein retention (%)					18	16					
Alkaline phosphatase (King-Armstrong units)	1.6	2.2	1.5	1.9	2.2	0.8		2.8	1.9	11.4	
Serum chloride (mEq/liter)		93									
Serum potassium (mEq/liter)		3.7	3.0	3.2							3.4
Gross and histopathologic evaluations											
Interstitial pneumonia	+++	+++	+++	+++	++	+	++	++	+++	+++	+++
Acute pneumonia	+		++	++						+	
Interstitial nephritis	++	++	+++		+++						
Hypocellularity, lymphoid tissues		+	++	+							
Testes degeneration				+							
Dermal lesions											
Ulceration, paw pads	+	+	+	+	+		+	+	+	+	
Onychoptosis		+	+	+	+				+		

TABLE 2.—Toxicity following iv bleomycin injection (0.625 mg/kg every 4th day) in dogs*—Continued

Dog No. and sex	681 M	678 F	666 M	673 M	685 M	686 F	687 M	695 M	684 F	624 F	625 M
Dermatitis, tail tip	+	+	+	+	+			+	+	+	+
Decubital ulcers at pressure points	+	+	+	+							
Focal alopecia	+	+		+	+					+	+
Fissuring and cracking, planum nasale			+								
Loss of whiskers and eyelashes	+	+	+								
Ulcer, frenulum linguae	+	+	+	+							

*NT = not tested. Toxicity rating: (+) = slight; + = moderate; ++ = marked; +++ = severe; ++++ = very severe; no entry = no toxicity.

†Incidental death was related to barbiturate anesthesia.

‡All blood values show significant toxic changes for that particular dog. Empty spaces indicate normal values.

Blood determinations	Mean	Range
Lymphocytes	2800	1700–3900
Blood urea nitrogen	14	>20
Sulfobromophthalein	6.8	>15
Alkaline phosphatase	4.4	2–7
Serum chloride	116.2	109–120
Serum potassium	4.7	4–5.4

TABLE 3.—Bleomycin (0.625 mg/kg iv every 4th day): temporal occurrence of weight loss, dermal lesions, and pulmonary changes*

Dog No. and sex	No. of treatments	Condition	Dermal lesion		Weight loss	Radiographic lesions	Decreased lung compliance†	
			Site	Day of appearance	(Day of appearance)	(Day of appearance)	Occurrence	Day of appearance
681 M	108	Poor	Paws	69	162	292	+	ND
678 F	103	Poor	Paws	53	240	230	+	ND
666 F	96	Poor	Paws	69	100	215	+	ND
673 M	94	Died	Paws	32	344	202	+	ND
685 M	46	Poor	Tail	60	53	183	+	64
686 F	34		None		None	None	0	
687 M	34		Paws	35	None	209	0	
695 M	34		Paws	37	46	None	0	
684 F	32		Tail	38	None	NT	+	70
624 F	31	Died	Tail	70	85	98	NT	ND
625 M	31		Tail	53	184	None	NT	ND

* Note absence of correlation between appearance of skin lesions and detection of radiographic lung opacities.

† + = Decrease; 0 = no decrease; NT = not tested; ND = not determined; no entry = no toxicity.

tilation, barbiturate overdosage, and/or increased sensitivity to the barbiturate.

Weight loss was generally dose related. Dogs treated with 94–108 doses developed a weight loss of 14%–34%. They became weak, barely capable of maintaining a standing position, and had an undernourished to emaciated appearance. Five of the seven other dogs also showed a loss of body weight (5%–18%).

In dogs without pulmonary complications, their poor general condition appeared to be associated with severe loss of body weight, azotemia, or both. The dog treated with 46 doses (No. 685 M) showed reversibility in his volume-pressure configuration following termination of treatment (fig 1), but he had azotemia (82.6 mg/100 ml) (table 2) and histopathologic evidence of a severe, chronic interstitial nephritis. Treatment for 12½–14 months caused chronic interstitial nephritis in three of four dogs (table 2).

A temporary moderate decrease of serum chloride or potassium was present in four of 11 dogs, and a reversible moderate increase of sulfobromophthalein retention was present in two of 11 dogs. Alkaline phosphatase was decreased in eight of 11 dogs and increased in one of 11 dogs. Intermittent, slight, or very slight lymphopenia occurred in seven of 11 dogs, and three of six dogs that were necropsied after termination of treatment showed a moderate or marked hypocellularity of the lymphoid tissues.

Dermal lesions occurred in ten of 11 dogs (table 2). They represented the first obvious signs of bleomycin-induced toxicity as reported in previous studies (25). These lesions appeared between 1 and 2 months after the onset of treatment, ie, after a minimal cumulated dose of 5 mg/kg (100 mg/m^2) (table 3). Dermal lesions were first manifested by fissures that appeared in the paw pads and progressed to skin ulcerations or by alopecia and ulcerative exudative dermatitis that affected the distal 2–5 cm of the tail. Subsequent lesions included loosening of the toe nails, onychoptosis, alopecia, and skin ulcers at pressure points on feet and legs. Skin lesions healed during continuation of bleomycin treatment, while new lesions erupted. Dogs treated for 12½–14 months also showed fissuring and cracking of the planum nasale, loss of whiskers and eyelashes, and ulcerations at the base of the tongue in the area of the frenulum linguae of approximately 10 mm in diameter.

Gross and microscopic examination of the intercostal and costochondral joints failed to reveal any immobility or fibrosis and the chest wall appeared to retain its mobility.

DISCUSSION

The marked or severe reduction in lung volume for a given pressure and the appearance of interstitial pneumonia and fibrosis in as much as 30%–40% of the cross-sectional area of the lung tissue indicated that intensive bleomycin treatment might be associated with a pattern of restrictive lung disease with decreased compliance in susceptible dogs. In animals necropsied immediately after termination of treatment the severity of lung histopathology was directly related to the duration of treatment. The incidence of radiographic lung lesions was parallel with the severity of morphologic changes. Changes in the elasticity of the chest wall or diaphragm could possibly have contributed to the altered volume-pressure curves. However, histopathologic examination did not verify this possibility.

Previous studies of bleomycin treatment in dogs demonstrated that a minimum treatment duration of 1–2 months was necessary for the appearance of morphologic manifestations of interstitial pneumonia and that pulmonary lesions usually progressed during a posttreatment observation period of 1–2 months (18). Following intensive treatment, pleural scarring and fibrosis became more severe than in earlier less extended treatment schedules. Our present observations on one dog (No. 685 M), that showed a decrease in lung and chest wall compliance during treatment followed by partial recovery during posttreatment, suggest that in some situations there can be some resolution of physiologic and pathologic parameters.

Partial recovery of altered pulmonary function was further supported by observations from a study by Valicenti et al (26) carried out in conjunction with the present investigation. Groups of two dogs that were injected

iv with 22 daily doses of bleomycin at 0.625 mg/kg or 11 daily doses at 1.25 mg/kg (every fourth day) developed reversible reduction in total lung capacity, functional residual capacity, and single breath diffusing capacity, while decrease in static lung compliance was irreversible.

In one of our dogs a decrease in static lung and chest wall compliance appeared within 70 days, ie, after 16 bleomycin treatments with a cumulated dose of 11.25 mg/kg (225 mg/m^2). Treatment with the same dose of 225 mg/m^2 in a phase I clinical trial in man led to extensive interstitial pneumonia (12).

It is not fully explained why some dogs had serious early pathologic and physiologic alterations while others required longer periods of drug exposure before toxic reactions appeared. Such a variation among one strain of dogs might also be anticipated in man. This would strongly support the necessity for clinical studies to include serial pulmonary function studies to differentiate susceptible patients from more tolerant patients.

Mortality appeared not to be dose related; of the two dogs that died, one died during early treatment (No. 624 F) and the other died after protracted treatment (No. 673 M). Death resulted from focal acute pneumonia superimposed on the interstitial pneumonia. Interstitial nephritis, another severe toxic manifestation of protracted treatment, led to a blood urea nitrogen value of 82.6 mg/100 ml in one animal (No. 685 M) who was killed 309 days after the onset of treatment. This kidney lesion may have progressed to uremia and death if the animal had been kept for a longer post-treatment observation period. The minimum lethal treatment for intensive treatment was 31 doses of 0.625 mg/kg (a cumulated dose of 19.375 mg [387.5 mg/m^2]), whereas in subacute toxicity studies, the minimum lethal treatment was 11 doses of 2.5 mg/kg (a cumulated dose of 27.5 mg/kg [550 mg/m^2]) (18).

Intermittent lymphopenia, hypocellularity of the lymphoid tissues, and absence of pyrexia in dogs with acute bronchopneumonia could result from immunosuppressive effects of repeated bleomycin treatment. However, signs of immunosuppression were not noted in this or previous studies (18,19).

The present observations also suggested that onset and severity of dermal toxic manifestations were not correlated with onset and severity of lung lesions (table 3). The development of skin lesions appeared to depend mostly on the physical activity of particular animals with subsequent exposure of the skin to trauma and friction.

Recent observations by Heyman et al (27) have demonstrated that bleomycin treatment of dogs (14 doses every fourth day at 5 mg/kg) induces a decrease in serum zinc levels to 50% of control values. Decreased serum zinc levels are associated with integumentary lesions and zinc deficiency could then be responsible for the increased vulnerability of the tissues in bleomycin-treated dogs to trauma and subsequent inflammation. The observation that eight of 11 dogs in this study reacted with a decrease in serum alkaline phosphatase, which requires zinc as a co-enzyme factor, appears to support the conclusion that repeated treatment with a relatively small dose of 0.625 mg/kg could have induced a decrease in serum zinc levels. Studies in progress will further clarify the importance of serum zinc levels in bleomycin toxicity.

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Microbiological Assay and Tissue Distribution of Mithramycin (NSC-24559) in Mice [1,2,3]

Carolyn Woolley and Robert F. Pittillo [4,5]

SUMMARY

A sensitive, precise, microbiological assay has been developed for use in evaluating the distribution and concentrations of mithramycin, an effective, antitumor agent, in the tissues of normal mice.

[Cancer Chemother Rep 58:311–316, 1974]

Mithramycin is an antibiotic produced by *Streptomyces plicatus* (1) which reportedly has antineoplastic activity against a variety of experimental neoplasms (1–4). It was clinically evaluated against many human malignancies (5–7) and has been reported to be of considerable value in the treatment of embryonal carcinoma (8), primary tumors of the central nervous system (9–11), and a variety of testicular cancers (12–15). Mithramycin (fig 1) is closely related (structurally) to chromomycin A_3 and olivomycin A (16).[6] Mithramycin is recognized as a specific inhibitor of RNA synthesis in vitro and in vivo (17). The reports on the mode of action, pharmacology, and toxicology have been reviewed (18). A microbiological assay was reportedly used in the original isolation of mithramycin, but no details of the techniques were published (1). Another microbiological assay has been described (19) for use in purity and potency tests and in the preparation of mithramycin for medicinal uses. However, currently used therapeutic drug levels of 25–50 μg/kg/day for 5 days, administered intravenously (iv) (15), were the criteria for the development of a more sensitive and defined microbiological assay. The purpose of this report is to describe a microbiological assay with sufficient sensitivity to be potentially applicable to clinical studies,

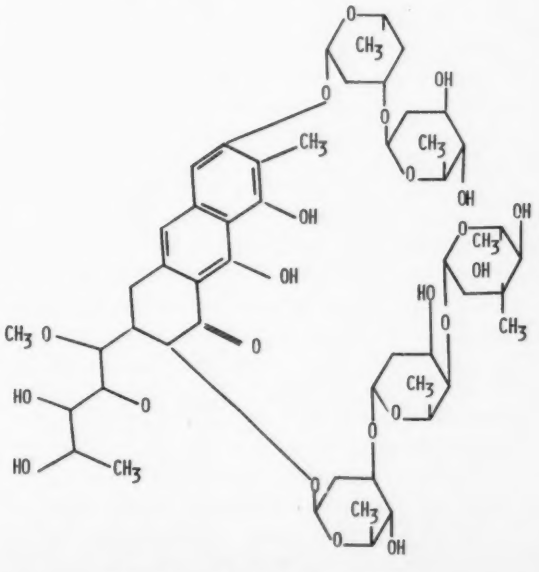

FIGURE 1.—Structure of mithramycin.

[1] Received July 10, 1973; revised Nov 4, 1973; accepted Nov 29, 1973.

[2] Supported by contract PH43-65-654 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] NSC-24559: CAS reg. No. 18378-89-7. Supplied by Drug Research and Development, DCT, NCI. Supplied by Drug Research and Development, DCT, NCI.

[4] Kettering-Meyer Laboratories, Southern Research Institute, Birmingham, Ala.

[5] The advice and support of J.A.R. Mead and L. B. Mellett and the technical assistance of Penhryn Bailey, Sherry Burris, and Diane Billings are gratefully acknowledged.

[6] Chromomycin A_3: NSC-58514; CAS reg. No. 7059-24-7.

Olivomycin A: NSC-76411; CAS reg. No. 6988-58-5.

and to report the physiologic disposition of mithramycin in normal mice at intervals following iv injection of single doses of this agent.

MATERIALS AND METHODS

Previously described methods were used to select an appropriate microorganism for the assay of mithramycin (20) in biologic materials. *Sarcina lutea* ATCC 9341 was selected as the assay organism. Tryptic Soy medium (Difco Laboratories) was used for culture maintenance, for inocula preparation, and for the preparation of the assay plates. Stationary broth cultures of *Sarcina lutea* ATCC 9341 were grown for 16–18 hours at 37 C. The cells were washed three times by centrifugation in sterile saline (0.85% NaCl), resuspended in sterile saline, and adjusted to 0.7 optical density units (1.6×10^8 cells/ml) in a Bausch & Lomb Spectronic-20 colorimeter adjusted to 660 nm. This cell suspension was diluted 1:100 with sterile saline, and a 10-ml amount of this suspension was added to 1 liter of cooled (50 C) Tryptic Soy agar which was adjusted to pH 6.0. Six-ml amounts of the inoculated agar were dispensed into plastic petri plates (100 × 15 mm, Lab-Tex Products). A stock solution of mithramycin was prepared in sterile saline (0.85% NaCl) and diluted in saline so that when 0.08-ml portions of the various dilutions were pipetted onto filter paper discs, the following concentrations were obtained: 0.004, 0.006, 0.008, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1,0.2, 0.4, 0.6, 0.8, and 1.0 μg of mithramycin per disc. An additional mithramycin stock solution was prepared by dissolving the drug in freshly collected heparinized mouse blood and diluting with saline. Mithramycin standard curves (prepared with the drug dissolved in homogenates of mouse liver, kidney, spleen, and brain) yielded results identical to those obtained when the drug was dissolved in blood. Filter-paper discs (1.27 cm in diameter, No. 740-E, Carl Schleicher and Schuell Co., Keene, NH) were impregnated with 0.08 ml of the respective solutions which contained graded concentrations of the drug. The discs were placed on the surface of each seeded agar plate and pressed down securely with flamed forceps. All experimental samples (body fluids or tissue homogenates) and standards were tested in triplicate. After 24-hour preincubation refrigeration at 4 C, all plates were simultaneously incubated for 15–18 hours at 30 C.

The resulting zones of inhibition were measured and corrected as previously reported (21). The corrected mean diameters of the zones of inhibition surrounding the discs, which contained known concentrations of drug, were plotted on semilogarithmic graph paper: the zone sizes on the arithmetic scale and the drug concentrations per disc on the logarithmic scale. Standard curves were constructed through the points thus obtained by the method of least squares. In the experimental samples, drug concentrations were obtained by reading the ordinate of the blood standard curve which corresponded to the size of the corrected zones of inhibition surrounding the discs impregnated with the tissue homogenates or body fluids. Mixed sexes of BDF_1 mice, weighing 18–22 g, were used in all experiments. Immediately and at 15, 30, 60, 120, and 240 minutes after iv injection blood samples were removed by cardiac puncture of sacrificed mice. Additionally, the brain, kidney, liver, lung, and spleen were removed, weighed, and prepared for assay. Each organ was homogenized with 1 ml of saline in an Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn). Urinary samples were removed directly from the bladder of sacrificed mice.

RESULTS AND DISCUSSION

Representative standard inhibition curves of mithramycin dissolved in saline (0.85% NaCl), in mouse blood, and in serum are presented in figure 2. (The varying limits of assay sensitivity are due to the random selection of curves rather than to biochemical factors.)

A group of BDF_1 mice were injected with a single dose of 2.14 mg/kg of mithramycin iv. Whole blood and serum levels, as well as urinary excretion and tissue distribution, were determined in these mice (fig 3*A*). Peak levels were observed immediately after drug administration in the blood and serum (12 and 6 μg/ml respectively). These levels declined rapidly and no drug was detected later than 1 hour after drug administration. Urine concentrations were found at 15 and 30 minutes after injection (1.5

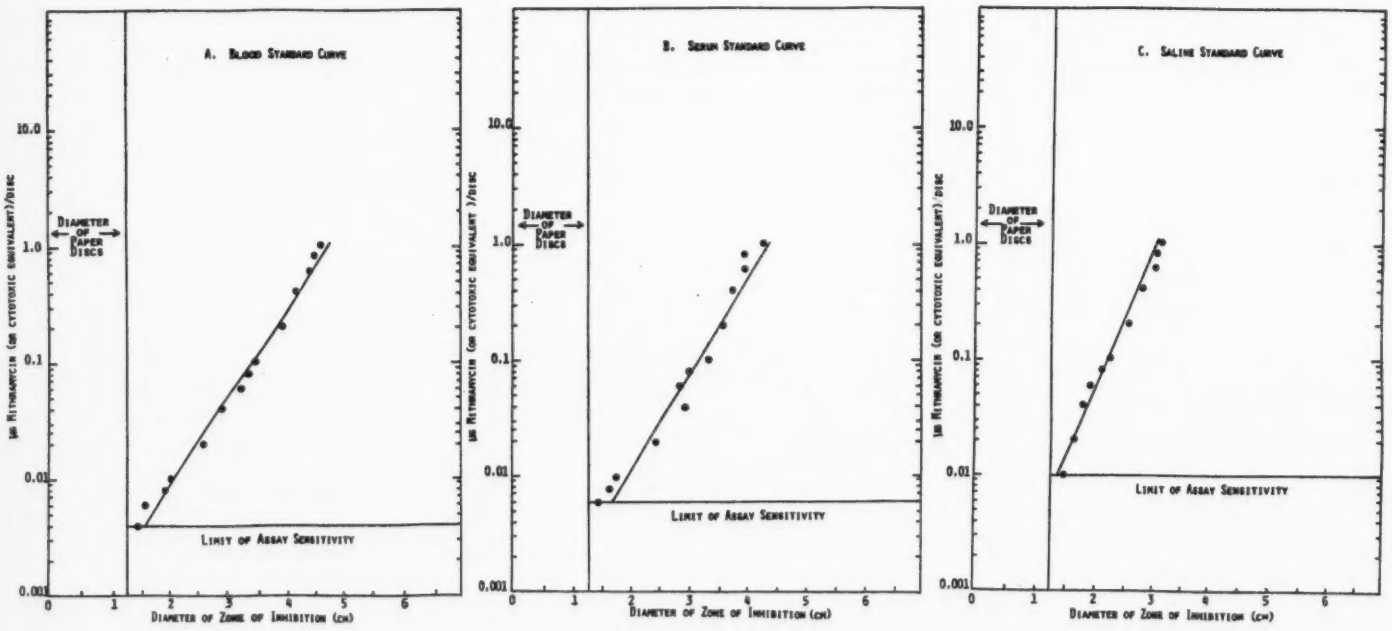

FIGURE 2.—Standard assay curves (constructed by method of least squares) for the logarithmic-ratio microbiological assay of mithramycin in tissues and body fluids of mice. Assay microorganism: *Sarcina lutea* ATCC 9341. (*A*) Blood standard curve: index of precision, 0.04. (*B*) Serum standard curve: index of precision, 0.04. (*C*) Saline standard curve: index of precision, 0.022.

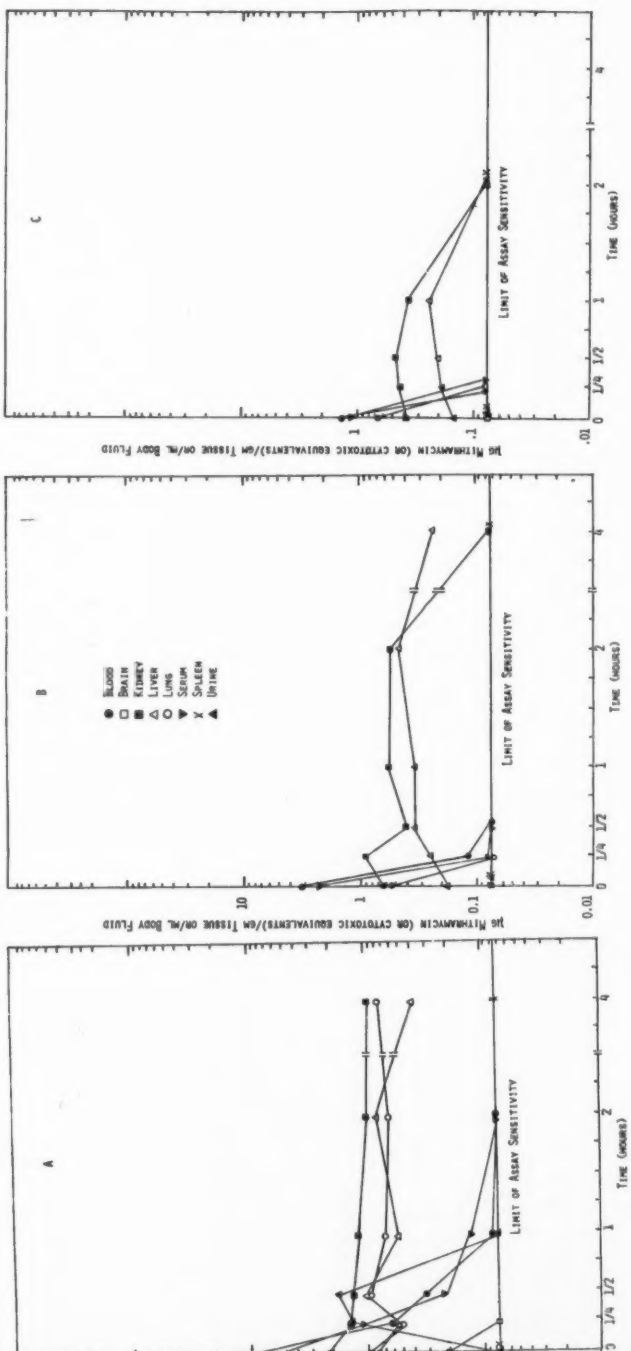

FIGURE 3.—Concentrations of mithramycin detected in tissues and body fluids of mice by microbiological assay. All blood levels represent the mean drug level of 5 mice. All serum and tissue levels represent analytical results of pooled samples from 5 mice. Assay microorganism: *Sarcina lutea* ATCC 9341. (*A*) Mice were injected with a single iv dose of 2.14 mg/kg (LD50) of drug. (*B*) Mice were injected with a single iv dose of 0.54 mg/kg (LD10) of drug. (*C*) Mice were injected with a single iv dose of 0.27 mg/kg (0.5 LD10) of drug.

and 1.8 μg/ml) but were not observed at other time intervals after injection.

Tissue distribution studies revealed accumulation of mithramycin in the kidney, lung, and liver of these mice through 4 hours. Peak levels (2.2, 0.95, and 0.8 μg/g respectively) occurred immediately after administration of the drug. A low level of 0.2 μg/g was detected in the brain immediately after injection but was not measurable at later time intervals. No drug was detected in the spleen at any time.

A second group of BDF_1 mice were administered a single dose of 0.54 mg/kg of mithramycin iv (fig 3*B*). Initial blood and serum levels (3.2 and 2.2 μg/ml respectively) declined rapidly and no levels were detected later than 15 minutes after injection. Tissue levels were detected only in the kidney, liver, and lung tissue. A maximum level of 0.95 μg/g was detected in the kidney at 15 minutes and measurable levels were obtained through 2 hours. Slightly lower levels were observed in the liver with a peak level of 0.35 μg/g occurring between 15 and 30 minutes. Some accumulation of drug was indicated through 4 hours in the liver. An initial level of 0.55 μg/g was observed in the lung but diminished by 15 minutes. No drug was detected in the brain, spleen, or urine at any time.

Tissue distribution and blood and serum levels were performed for a third dose of drug. BDF_1 mice were administered a single dose of 0.27 mg/kg of mithramycin iv (fig 3*C*). Initial blood levels (1.4 μg/ml) and serum levels (1.1 μg/ml) diminished rapidly. Tissue distribution studies revealed some accumulation of drug in the kidney and liver through 1 hour. A peak level of 0.48 μg/g occurred in the kidney at 30 minutes and no drug was detected in the kidney at 2 hours. An initial tissue level of 0.15 μg/g in the liver gradually increased to 0.24 μg/g at 1 hour and diminished by 2 hours. A tissue level of 0.68 μg/g was observed in the initial lung sample only. No measurable drug was detected at any time in the urine, brain, or spleen.

These results are in good general agreement with those reported by Kennedy et al (22) who did similar studies in mice using tritiated mithramycin. It may be noted, however, that in both studies no positive identification tests have been done to determine if mithramycin or some breakdown product is actually being measured. Such work is currently in progress.

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Effect of Reserpine (NSC-59272)-Induced Hypothermia on Lifespan in Mouse L1210 Leukemia [1,2,3]

Thomas P. Fondy, [4,5] Katherine L. Karker, [4,6] Carol Calcagnino, [4] and Cheryl A. Emlich [4,7]

SUMMARY

In order to employ in vivo model systems for the chemotherapeutic evaluation of agents that also happen to produce hypothermia in test animals, it is necessary that the effect of hypothermia itself on tumor growth or survival parameters be considered. Reserpine was used to produce a stable hypothermia in BDF_1 mice bearing L1210 ascites leukemia. Mice were maintained at ten different ambient temperatures ranging from 22 to 33 C and the percent increase in median and mean lifespan with respect to controls similarly maintained was determined. Increases in median and mean lifespan of 40%–50% were obtained by means of reserpine-induced hypothermia. Reserpine itself did not increase survival time when hypothermia was prevented by keeping the treated mice at elevated ambient temperature, under conditions in which reserpine alone was nontoxic. Hypothermia was a major contributing factor to the previously observed antileukemic effects of reserpine in the L1210 system and very likely in other murine tumor systems. Thus, any potential chemotherapeutic agent that also happens to produce hypothermia could give a very substantial positive result due to hypothermia alone. By use of an empirical relationship presented between survival parameters and either average temperature or a hypothermia parameter defined as temperature-time below controls, it is now possible to make a firm quantitative correction for effects due to hypothermia alone. A possible approach to detecting and monitoring drug-induced hypothermia in mass screening systems is suggested.

[Cancer Chemother Rep 58:317–324, 1974]

[1] Received Sept 14, 1973; revised Feb 15, 1974; accepted Feb 21, 1974.

[2] Supported by grant CA-10250 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Reserpine: CAS reg. No. 50–55–5; benz[*g*]indolo-[2,3-*a*]quinolizine-1-carboxylic acid, 1,2,3,4,4*a*,5,7,8,13,-13*b*,14,14*a*-dodecahydro-3-hydroxy-2,11-dimethoxy-, methyl ester, 3,4,5-trimethoxybenzoate; Serpasil. Supplied by CIBA Pharmaceutical Co., Summit, NJ.

[4] Department of Biology, Syracuse University, NY.

[5] Recipient of Public Health Service Research Career Development Award (CA-70332) from the NCI.

While studying the pharmacology of D- and L-1-fluorodeoxyglycerols and their 3-phosphate esters, a pronounced hypothermia was observed in BDF_1 mice.[8] Similar observations were also

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[7] We are grateful to Dr. William Bradner, Bristol Laboratories, for providing the L1210 leukemia line and for advice in the work.

[8] Fondy TP, Pero RW, Karker KL, et al. Synthesis of L-1-fluorodeoxyglycerol, its 3-phosphate ester, and effects of the L- and D-enantiomers in BDF_1 mice. J Med Chem, Aug 1974. In press.

made for 3-fluorodeoxy-D-sorbose [9] and 1-fluoro-3-hydroxyacetonephosphate (1). Hypothermia due to fluoroacetate had been mentioned earlier (2). At dose levels that could be employed in the evaluation of these fluoro-organics as cancer chemotherapeutic agents, body temperatures in BDF_1 mice were reduced to within 2 degrees of ambient. When hypothermia was prevented by maintaining an elevated ambient temperature, the toxicity of the fluoro-organics was substantially increased. As a result, in vivo tumor model systems could not be used for the chemotherapeutic evaluation of these compounds, or indeed of any compounds producing hypothermia under similar conditions in the host animals, until the effect of the hypothermia on tumor growth and survival in the model system was placed on a firm quantitative basis. Since we initially planned to evaluate the effects of the fluoro-organics in the L1210 mouse leukemia ascites system, we undertook to establish the effect of hypothermia on survival time in that system. Examination of various alternatives led us to select the plant alkaloid reserpine as a means of inducing a reasonably stable hypothermia required for evaluation of effects of hypothermia on the L1210 system. Earlier studies had shown a strong antileukemic action for reserpine in the solid L1210 system (3,4) and in Sarcoma 37 (5). Positive antileukemic effects of reserpine in L1210 ascites leukemia were thought to be affected only to a minor extent, if at all, by the reserpine-induced hypothermia (6–8). However, it did not prove possible to separate the effects of reserpine on solid and ascites lymphoma L-1, Sarcoma 37, and Sarcoma 180 (9), nor on solid L1210 leukemia (10) from its generalized depressant effects. It was concluded that the anticancer effects of reserpine in the murine cancer systems studied were possibly (10) or probably (9) nonspecific, and were not due to an intrinsic anticancer action of the drug (11).

During the course of employing reserpine as a means of quantitating the effect of drug-induced hypothermia in L1210 leukemia, we have established that hypothermia has a very substantial effect on survival parameters in L1210 ascites leukemia, demonstrating that a large part, if not all, of the anticancer effects previously observed with reserpine in mice were due to the profound hypothermia induced. These studies point out the need for considering drug-induced hypothermia as a source of positives in murine cancer chemotherapy screening systems, and they provide a convenient means of correcting for the hypothermic effect on survival parameters in the L1210 ascites system. A possible means of observing and quantitating hypothermia in mass screening programs is suggested.

[9] Rao GV, Fondy TP, and Hall LD. Deoxyfluoroketohexoses: 4-deoxy-4-fluoro-D-sorbose and fructose and 3-deoxy-3-fluoro-D-sorbose. Submitted to *Carbohydrate Research.*

MATERIALS AND METHODS

L1210 leukemia was obtained from Dr. William Bradner, Bristol Laboratories, Syracuse, New York, and was maintained in DBA/2 female mice. Ascites tumor cells were counted in a hemocytometer and diluted in sterile saline to 10^7 cells/ml; 0.1 ml (10^6 cells) was injected intraperitoneally (ip) into female BDF_1 hybrids (C57BL/6 female × DBA/2 male), 8–10 weeks of age, weighing 19–23 g.

Reserpine was obtained as a solution at 2.5 mg/ml in sterile water and was further diluted 1:10 in sterile water (it is insoluble in saline). Reserpine was administered ip at either 1 or 2 mg/kg as detailed under Results.

Experiments were conducted at ambient temperatures (± 0.5 C) of 22, 24, 25, 26, 27, 28, 29, 30, 31, and 33 C. At each ambient temperature, three groups of eight mice each were employed. One group received L1210 cells and reserpine, the second received L1210 cells and sterile water injections, and the third group consisted of drug controls receiving reserpine but no ascites cells. Deaths were checked every 6 hours and survival times were assigned as midway between the time the animal was found dead and the last time it was observed alive. Thus, survival times were known to within 0.1 day. Animals receiving both L1210 and reserpine were autopsied and the presence of L1210 leukemia was confirmed by the ascites fluid and by the extensive vascularization of the peritoneal cavity.

The initial dose of reserpine was adminis-

tered 24 hours after L1210 transplant at a dose level designed to obtain a rapid drop in body temperature approaching ambient. Subsequent doses of reserpine were spaced to maintain as stably as possible a subnormal body temperature (see Results) near ambient at least through Day 5 after tumor transplant. Mouse temperatures were monitored at intervals of 8 hours with a Tele-thermometer (Yellow Springs Instrument Co.) with thermocouple probe No. 423 inserted rectally to approximately 1 cm and allowed to equilibrate for 45 seconds.

RESULTS

Figures 1 and 2 show the body temperatures achieved in the various groups of mice by the combination of reserpine and a series of ten different ambient temperatures ranging from 22 to 33 C. The experiments were carried out in three sets: at 31, 27, and 25 C; at 33, 29, and 22 C; and at 30, 28, 26, and 24 C. Slightly different dose schedules of reserpine were used in each of the three sets based on experience with the preceding set in an attempt to improve the stability of the subnormal temperatures achieved. The extent of hypothermia was quantitated by determination of the area under the curve between L1210 controls and the group that received L1210 and reserpine. This area is designated "temperature-time below controls" (TtBC) and has the units of degrees centigrade × hours. The level of hypothermia was also defined by the average temperature ($\bar{T}$) for the reserpine-treated L1210 mice. The effect of hypothermia on mean and median survival of leukemic mice is shown in table 1. Both mean and median lifespan were used in

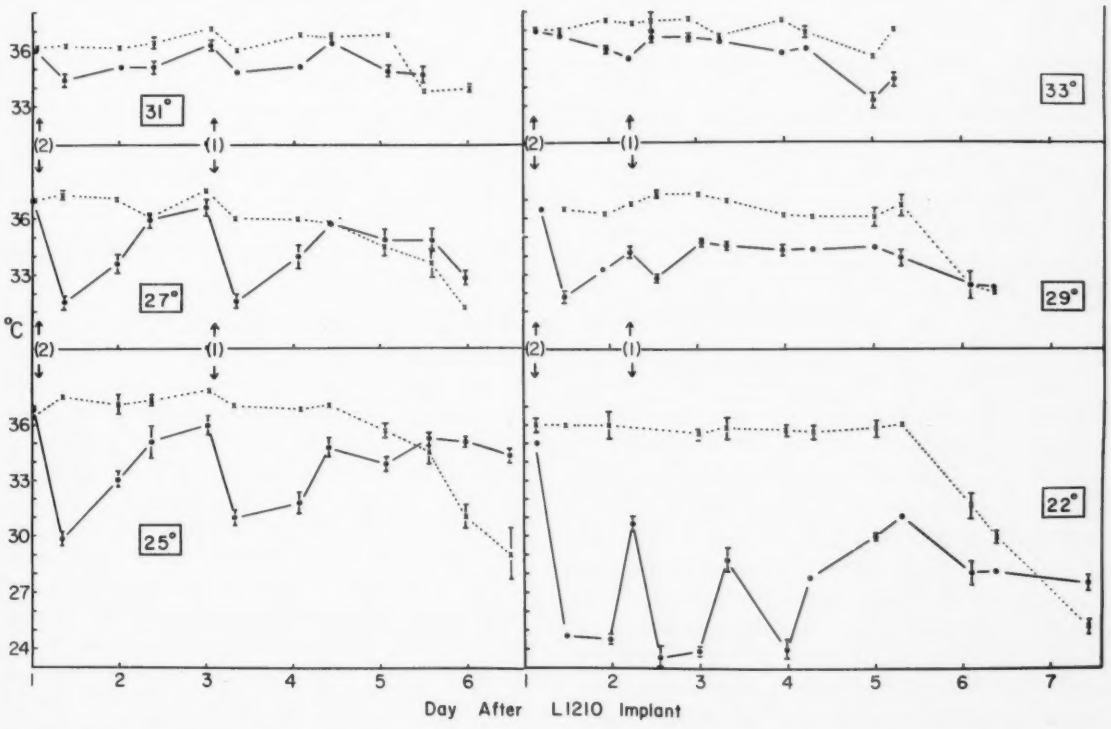

FIGURE 1.—Temperatures (± standard error of the mean) of mice (8 per group) maintained at different ambient temperatures and receiving 10^6 L1210 cells on Day 0 followed by reserpine (————) or saline (. . . . .). Doses of reserpine in mg/kg are shown in parentheses and the time of dosing is shown by the double arrow. Reserpine-treated, non-L1210 control groups have not been included in order to simplify the figure. For typical behavior of reserpine controls, *see* fig 2.

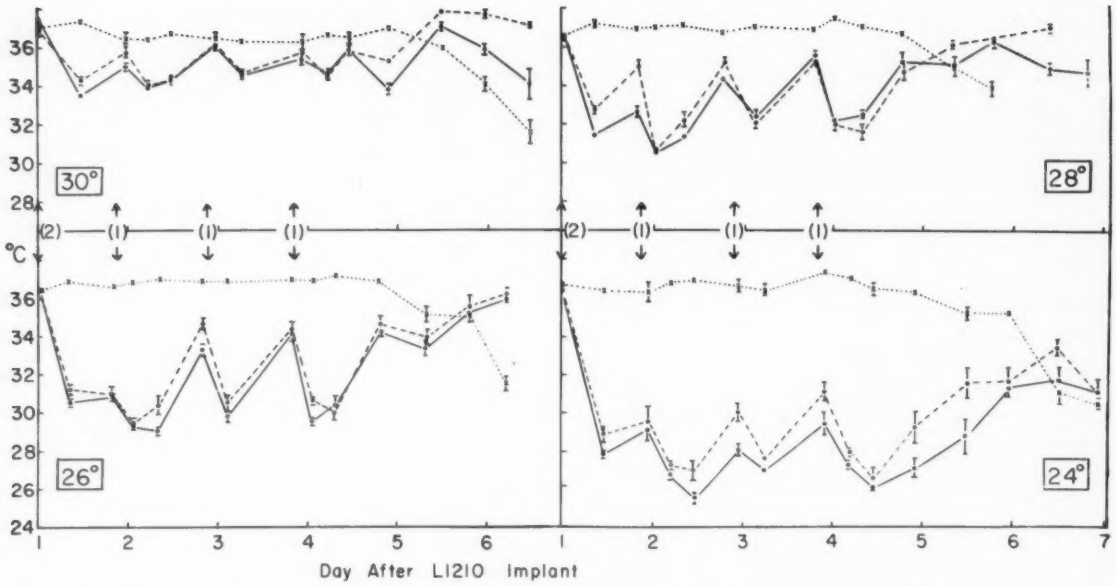

FIGURE 2.—Temperatures (± standard error of the mean) of mice (8 per group) maintained at different ambient temperatures and receiving 10^6 L1210 cells on Day 0 followed by reserpine (————) or saline (. . . . .). Reserpine-treated, non-L1210 controls (. . . . .). Doses of reserpine in mg/kg are shown in parentheses and the time of dosing is shown by the double arrow.

this work in order to facilitate application of the correction factors to other hypothermia-producing drugs where one or the other parameter may be in use.

Figure 3*A* represents the percent increase in median and mean lifespan as a function of the TtBC. Figure 3*B* relates the percent increase in median and mean lifespan to the average temperatures of the reserpine-treated L1210 mice. Only at 22 C ambient were there deaths in the reserpine control group, which introduces uncertainty into the determination of the effect of hypothermia on prolongation of lifespan in the mice in that group. It is apparent that for dose schedules applicable to the examination of the effect of fluoro-organics on L1210 leukemia [8] there is significant prolongation of lifespan which is due to hypothermia alone. The correction for such hypothermic effect for these or any other chemotherapeutic agents which lower body temperature in mice can now be made by reference to figure 3.

DISCUSSION

Corrections for Hypothermic Effect on Survival Parameters

Figure 3 illustrates that above an average body temperature of 33 C or below a TtBC of 400° hours there is an approximate linear relationship between these parameters and an increase in either median or mean lifespan. There is only a slight deviation from linearity for the percent increase in mean lifespan over the entire range of temperatures examined. In the case of median lifespan, there is a substantial deviation from linearity at lower mouse temperatures. These relationships are of use not only in correcting for the effect of hypothermia on the survival parameters, but also for establishing the point at which drastic variation in mouse temperatures might introduce errors in the corrections. It is apparent that where the minimum temperatures do not go below approximately 33 C, there should be no important difference in percent increase in

TABLE 1.—Survival during reserpine-induced hypothermia in BDF_1 mice bearing L1210 ascites leukemia*

Ambient temperature (C)	Temperature-time below controls (°C × hrs)	Mean temperature (±SEM) (C)	Reserpine-treated mice with L1210		Control mice with L1210		Reserpine-treated controls (survivors beyond Day 20/total No. treated)
			Mean survival (days ± SEM)	Median survival (range in days)	Mean survival (days ± SEM)	Median survival (range in days)	
22	883	28.3 ± 0.6	11.1 ± 0.6†	10.5(9.4–14.0)†	7.5 ± 0.2	7.4(6.8–8.6)	0/8‡
24	983	28.4 ± 0.4	10.6 ± 1.1†	9.2(7.7–15.2)†	7.1 ± 0.1	7.0(6.9–7.7)	8/8
25	382	33.6 ± 0.3	7.7 ± 0.3†	7.5(6.9–9.0)†	6.3 ± 0.1	6.3(6.0–7.0)	8/8
26	553	31.6 ± 0.3	8.7 ± 0.2	8.6(7.9–9.8)	6.9 ± 0.1	6.7(6.5–7.6)	7/8
27	213	34.6 ± 0.2	6.8 ± 0.1†	7.0(6.6–7.0)†	6.2 ± 0.1	6.4(5.7–6.6)	8/8
28	374	33.1 ± 0.2	7.5 ± 0.1†	7.5(7.2–7.9)†	6.2 ± 0.1	6.1(5.9–6.5)	8/8
29	228	34.2 ± 0.2	6.5 ± 0.1†	6.4(6.1–7.2)†	5.8 ± 0.1	5.6(5.5–6.4)	8/8
30	173	35.1 ± 0.1	6.5 ± 0.1	6.5(6.3–6.9)	6.1 ± 0.1	6.1(5.9–6.4)	8/8
31	122	35.3 ± 0.3	5.6 ± 0.1	5.7(5.2–5.8)	5.7 ± 0.1	5.7(5.2–6.0)	8/8
33	115	35.7 ± 0.3	5.5 ± 0.1	5.6(4.6–6.0)	5.8 ± 0.1	5.6(5.5–6.4)	8/8

*Female mice, 19–23 g, 8 per group, received 10^6 ascites cells ip on Day 0. Reserpine doses and scheduling are shown in figs 1 and 2 and consisted of either 3 or 5 mg/kg (total dose) given over the length of the experiment.

†One drug death before Day 4.6 is excluded.

‡Mean survival = 11.1 days ± 0.9 SEM; median survival = 10.5 days (range, 7.2–14.0); both figures exclude 1 drug death before Day 4.6.

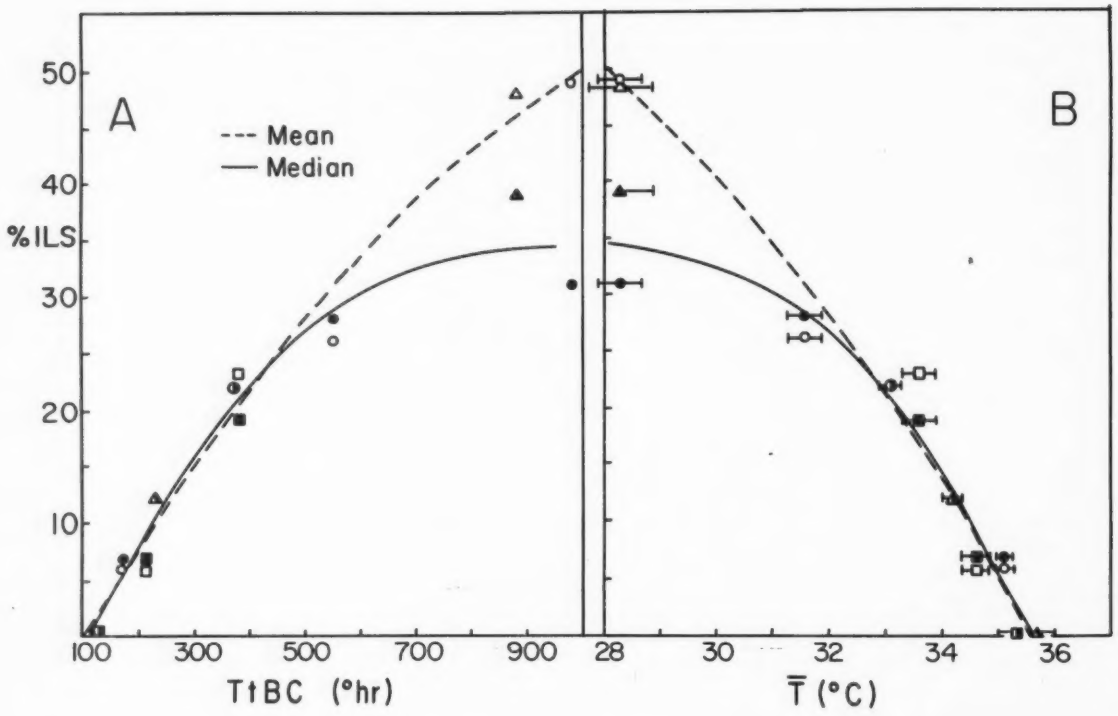

FIGURE 3.—Percent increase in lifespan (%ILS), median (————) and mean (.....), for reserpine-treated L1210 mice. (*A*) As a function of TtBC in °hrs (area between reserpine-treated and saline-treated L1210 mice in figs 1 and 2 to Day 6). Standard error of the mean areas were 10% at 500° hrs. (*B*) As a function of average temperature ($\bar{T}$) ± standard error of the mean of reserpine-treated L1210 mice (figs 1 and 2) to Day 6. Open symbols refer to mean survival, filled symbols to median survival. Where these parameters are identical, half-filled symbols are used.

□ = Experiments at ambient 31, 27, or 25 C (fig 1).
△ = Experiments at ambient 33, 29, or 22 C (fig 1).
○ = Experiments at ambient 30, 28, 26, or 24 C (fig 2).
The curves are computer-fitted using the least squares criterion of fit for a polynomial of the second degree.

survival between groups which are uniformly maintained at a given body temperature and those which achieve the same average body temperature but with wide variation in temperature as a function of time. This effect is clearly seen in the comparison of the increase in survival parameters for the groups shown in figure 1 (ambient 27 C) and the group shown in figure 2 (ambient 30 C). In the latter group the temperature was maintained at a fairly constant level, while in the former group there was considerable variation in temperature. In both cases the TtBC and the average temperature were approximately the same (because the 27-C group received a total of 3 mg/kg of reserpine in two doses, while the 30-C group received a total of 5 mg/kg in four doses) and the survival parameters were identical.

Effect of Ambient Temperature on Survival

In examining both control and reserpine-treated L1210 mice at a variety of ambient temperatures, we observed that the terminal stages of the disease were considerably shortened at the higher ambient temperatures. With ambient temperature at 22 C, the median survival of the L1210 control group given 10^6

cells was 7.5 days, at 28 C it was 6.1 days, and at 33 C it was 5.6 days. The reason for this effect is apparent by reference to figures 1 and 2. There is a very substantial drop in body temperature in the L1210 mice beginning around Day 5 after tumor implantation. In mice maintained at lower ambient temperatures the hypothermia induced by L1210 leukemia itself prolongs survival. In groups maintained at elevated ambient temperatures this terminal hypothermia is prevented and survival is substantially reduced.

Effect of Reserpine on L1210 Leukemia

It is apparent that reserpine itself, exclusive of the hypothermia it induces, has no effect on prolonging survival in L1210 leukemia at the dose schedules used in this study. When hypothermia is prevented by maintenance of ambient temperature at 31 or 33 C there is no increase in median or mean survival. Thus the different total dosages of reserpine and the different dose schedules used in figures 1 and 2 do not differentially affect the survival parameters. The percent increase in mean lifespan previously observed (6) in L1210 ascites leukemia using reserpine at approximately the same total dosage used in this work gave a value of 12%–26%, well within the range expected due to hypothermia alone. The fact that chlorpromazine, another hypothermia-inducing drug, also produced a 19% increase in mean survival in the earlier study (6) again suggests an effect in the range of 20% under these conditions due to hypothermia alone. Whether all of the anticancer effects observed with reserpine at higher dosages and in other test systems (3–5, 8–10) can be attributed to hypothermia alone cannot be established directly from the correction factors introduced here. However, it is apparent that hypothermia has a very substantial effect on tumor cell growth rate and survival parameters, and thus becomes a prime candidate to be the nonspecific, host-mediated, antileukemic factor previously suggested as operating in the anticancer effects of reserpine in mice (9–11).

Alternative Approaches to Chemotherapeutic Evaluation of Agents Which Induce Hypothermia

In addition to the use of hypothermic corrections as developed in this work, there are several other alternatives for the chemotherapeutic assessment of agents which also happen to induce hypothermia. If the drug-induced hypothermia can be reduced or eliminated by maintaining the test animals at elevated ambient temperature without excessive stress or enhanced toxicity, effects due to hypothermia alone can be resolved. Maintaining elevated ambient temperature may not be possible or practical. In such cases use of tumor model systems with longer survival times would tend to minimize the effect of hypothermia at a given total drug dose, since the average temperature for the duration of tumor growth would be closer to that of untreated controls. With rapidly growing models such as the L1210 system, the use of lower numbers of cells in the challenge dose would also tend to reduce the effect of hypothermia induced at a given dose level.

Tumor model systems employing strains of mice that are less susceptible to the hypothermic effect are another alternative. In the case of the fluoro-organics mentioned earlier,[10] this condition is met by use of the various Ehrlich cell lines in non-inbred HAICR mice.

The most useful, and indeed essential, methods for evaluating potential chemotherapeutic agents that also induce hypothermia are the combined in vitro-in vivo model systems employed by Giovanella, Lohman, and Heidelberger (12) and by Bekesi, Molnar, and Winzler (13). The temperature of dividing cancer cells exposed to the potential chemotherapeutic agent outside the test animal would be maintained independent of any hypothermic effects. Subsequently, the treated cells could be introduced into test animals without the introduction of a significant dose of the hypothermic agent.

Detection and Quantitation of Hypothermic Effects in Mass Cancer Chemotherapy Drug Screening Systems

The techniques employed in this work for first observing and then monitoring hypothermic effects on the laboratory scale are not ideal for application to large volume drug

[10] *See* footnote 8.

screening work. Agents which may be producing positives due to hypothermia in mice can be initially detected by observation of the activity patterns of the treated animals. Test animals that become inactive and lethargic and tend to huddle together are very likely to be suffering from substantial hypothermia. However, it is possible for animals with rectal temperatures 3 or 4 C below normal to exhibit apparently normal activity patterns. The use of liquid crystal thermography tape as a means of visually detecting and monitoring mouse skin temperature within 1 C recommends itself for application at both the research and mass screening levels.

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Cell-Mediated Response to a Mouse Leukemic Subline Antigenically Altered Following Drug Treatment In Vivo[1,2,3]

Angelo Nicolin,[4] Alfredo Bini,[4] Paola Franco,[4] and Abraham Goldin[5,6]

SUMMARY

The antigenic alteration of L1210 leukemia after treatment in vivo with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (L1210/Ha/DIC leukemia line) was demonstrated by a cell-mediated immune reaction in vitro. Spleen cells collected from CDF_1 mice immune to the L1210/Ha/DIC subline were specifically cytotoxic to the cells of the same leukemia subline, as evaluated by the ^{51}Cr-release technique, but showed little or no cytotoxic activity for a series of other tumor or normal cells from syngeneic or allogeneic donors. The lysis of target cells increased as the dose of the immunizing inoculum was increased. With decreasing ratio of the immune lymphocytes to the labeled target cells, there was a reduction in the lysis of target cells.
The specificity of the cytotoxic reaction was supported further by the inhibition of ^{51}Cr release exerted by the unlabeled L1210/Ha/DIC cells added to ^{51}Cr-labeled L1210/Ha/DIC cells incubated with immune lymphocytes. The specificity of this cell-mediated immune reaction favors the hypothesis that new antigen(s) could appear on L1210 leukemic cells following chemotherapeutic treatment in vivo.

[Cancer Chemother Rep 58:325–330, 1974]

The existence of tumor-associated antigens is by now well established both in experimental animals and man (1–4). This finding has important therapeutic implications, since the immunotherapeutic approaches to cancer are founded on the existence of antigenic differences between normal and neoplastic cells.

A series of studies (5–8) demonstrated the possibility that antigenic changes could occur in neoplastic cells as a result of chemotherapeutic treatment in vivo.

In some instances, a strong allograft reaction was observed when leukemic cells obtained after several transplant generations in drug-treated mice were inoculated into mice compatible with the parental leukemic cells. Furthermore, humoral antibodies, specific for the altered sublines but not for the parental leukemic line, were obtained (8,9).

Although the above studies favor the hypothesis that altered sublines are rejected by an immune mechanism, no direct evidence of a

[1] Received Sept 13, 1973; revised Jan 11, 1974; accepted Jan 29, 1974.

[2] Supported in part by "Centro di Farmacologia delle Infrastrutture," Consiglio Nazionale delle Ricerche, Rome, Italy.

[3] Drugs used in this study were supplied by Drug Research and Development (DR&D), Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

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[6] We wish to thank Mr. S. M. Poiley, Mammalian Genetics and Animal Production Section, DR&D, DCT, NCI, for breeding and providing the animals. We are also grateful to Dr. Enzo Bonmassar for his interest in the investigation and to Dr. M. I. Colnaghi for helpful suggestions concerning the cell-mediated cytotoxicity technique.

cellular immune response has been obtained. In the studies reported here specific cytotoxic activity associated with mouse spleen cells was found after rejection of a high inoculum of an L1210 leukemia subline obtained in animals treated with 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (NSC-45388).[7]

MATERIAL AND METHODS

Animals

Inbred C57BL/6J, C57BL/10 Sc Sn, DBA/2 Cr, BALB/c Cr, hybrid CDF_1 (DBA/2 Cr male × BALB/c female), and hybrid BDF_1 (DBA/2 Cr male × C57BL/6J female) mice of both sexes were used. The animals were 2–4 months old, weighed 20–30 g, and were maintained at standard laboratory conditions.

Tumors

L1210/Cr (NCI) and L5178Y lymphomas were supplied by Microbiological Associates, Inc., Bethesda, Maryland. L1210/Ha (Hauschka) was kindly furnished by Dr. E. Mihich, Roswell Park Memorial Institute, Buffalo, New York, and EL 4 and E male G2 were obtained through the courtesy of Dr. G. Della Porta, National Cancer Institute, Milan, Italy. L1210 and L5178Y were maintained by serial passage in DBA/2 Cr or CDF_1 mice. EL 4 and E male G2 were maintained in C57BL/6J or BDF_1 mice.

The NSC-45388-transformed subline, L1210/Ha/DIC, was developed in CDF_1 mice after daily intraperitoneal (ip) treatment with NSC-45388, 100 mg/kg, for three transplant generations as described earlier (10). L1210/Ha/DIC leukemia was maintained in CDF_1 mice previously immunosuppressed with cyclophosphamide[8] (200 mg/kg given ip 5 hours before tumor implantation).

Drugs

NSC-45388 was suspended in a chilled solution of carboxymethylcellulose protected from light. Cyclophosphamide was dissolved in saline and kept in an ice bath. The drug preparations were made immediately before use.

In Vitro Assay for Cell-Mediated Cytotoxicity

The technique described by Wigzell (11) and modified by Canty (12) was employed.

CDF_1 mice were inoculated ip with L1210/Ha/DIC viable cells and killed 10 days later. Normal nonleukemic mice were killed at the same time. When not specified, 10^7 spleen cells (attackers) suspended in 1 ml of medium 199 supplemented with 10% heat-inactivated fetal calf serum were incubated in 35-mm petri dishes (Falcon Plastic Co.) on a rocking platform with 0.05 ml of supplemented medium containing 5×10^4 ^{51}Cr-labeled cells (targets) in a 37 C incubator with a moist atmosphere of 10% CO_2–90% air for 4 hours. The ^{51}Cr released by target cells was counted in an automatic well-type scintillation counter (Nuclear Chicago Corp.).

Quadruplicate samples were used for each experimental group and the percent cytotoxicity for each group was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{\text{cpm } ^{51}\text{Cr released from incubated cells}}{\text{cpm } ^{51}\text{Cr released from frozen-thawed cells (4 times)}} \times 100.$$

Controls for each experiment included target cells incubated with normal unsensitized spleen cells. Experimental results were expressed as:

$$\% \text{ Cytotoxicity in experimental samples} - \% \text{ cytotoxicity in control samples.}$$

Assay for Inhibition of Cytotoxicity

The specificity of the cytotoxic reaction was checked by an inhibition assay as described by Ortiz de Landazuri et al (13).

To the incubating suspension of attacker: target cell ratio of 200:1, unlabeled cells were added to give a final attacker: target cell ratio of 66:1.

RESULTS

Data not reported here confirmed the previous findings obtained in our laboratory (10) that CDF_1 mice, originally compatible with the L1210/Ha leukemia, can reject 10^7 L1210/Ha/DIC viable cells inoculated ip. As reported in table 1, 10 days after immunization against NSC-45388-transformed L1210/Ha/DIC leukemia, spleen cells of CDF_1 mice

[7] NSC-45388: CAS reg. No. 4342-03-4; DIC.

[8] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

elicited strong specific cytotoxic activity against the same tumor line. The same immune spleen lymphocytes did not show any cytotoxic activity against a series of normal or leukemic cells syngeneic or allogeneic with DBA/2 mice. However, the L1210/Ha cells were partially lysed by spleen lymphocytes immune against L1210/Ha/DIC cells. It is conceivable that L1210/Ha leukemic cells carry some transplantation antigen retained by L1210/Ha/DIC leukemia, which could account for the observed cross reactivity.

Figure 1 shows the percentage of ^{51}Cr released from L1210/Ha/DIC cells incubated with immune spleen cells collected at various intervals after immunization. As described for Gross virus-induced lymphoma under similar experimental conditions (14), the cytotoxic activity of spleen lymphocytes was detectable from Day 8 to Day 12 after antigen administration, with a peak on Day 10. Furthermore, cellular cytotoxicity increased as the dose of immunizing inoculum was increased (fig 1).

Reduction of target cell lysis occurred as the attacker:target cell ratio was decreased (fig

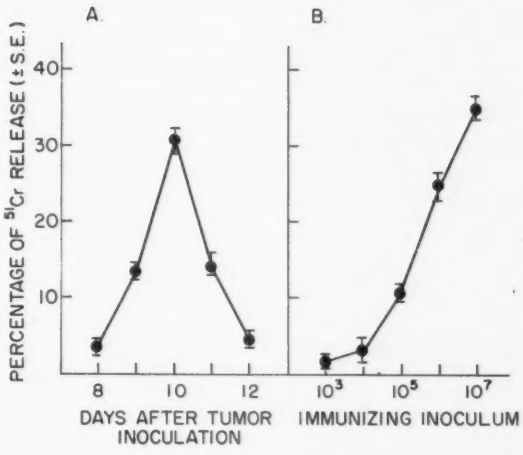

FIGURE 1.—*A*. Kinetics of cell-mediated cytotoxic response to L1210/Ha/DIC leukemia (10^7 cells ip). *B*. Relationship between the number of L1210/Ha/DIC leukemic cells in the immunizing inoculum and the cytotoxic response.

TABLE 1.—Cytotoxic activity of spleen cells from CDF_1 mice 10 days after inoculation of 10^6 viable L1210/Ha/DIC cells*

Target cells†	Histocompatibility relationship with DBA/2 strain	^{51}Cr release ± SE (%)
L1210/Ha/DIC	Syngeneic	31±2.8
L1210/Ha	Syngeneic	14±1.7
L1210/Cr	Syngeneic	1±0.3
DBA/2 thymus	Syngeneic	2±0.3
CDF_1 thymus	Syngeneic	1±0.2
L5178Y	Syngeneic	2±0.4
BALB/c thymus	Allogeneic	1±0.1
C57BL/10 thymus	Allogeneic	2±0.3
EL 4	Allogeneic	0±0.1
E male G2	Allogeneic	3±0.3

*The attacker:target cell ratio was 200:1. All values for these and the following assays are the means of quadruplicate incubations. For these and the following experiments CDF_1 male mice were the donors of normal and immune splenocytes.

†Spontaneous release of ^{51}Cr of various labeled target cells ranged from 15% to 20%.

2), providing further support for an immunologic basis of the cell-mediated cytotoxicity. This was observed with an experimental design in which a fixed number of ^{51}Cr-labeled L1210/Ha/DIC target cells was added to a decreasing number of immune attacker cells in the incubation system. Parallel results were obtained in an experiment where an increasing number of unlabeled L1210/Ha/DIC target cells was added to reduce the original 200:1 attacker:target cell ratio.

The specificity of L1210/Ha/DIC antigen(s) was also evaluated by the assay for inhibition of cytotoxicity: 10^5 unlabeled normal or leukemic lymphoid cells were added to the 200:1 attacker:target cell ratio. If the unlabeled cells carry some common antigen(s) with the L1210/Ha/DIC cells (labeled target cells), they will divert attacker cells from their lytic activity against the ^{51}Cr-labeled cells, resulting in a reduction of ^{51}Cr release. Table 2 summarizes the data of this inhibition assay. The unlabeled L1210/Ha/DIC cells reduced the

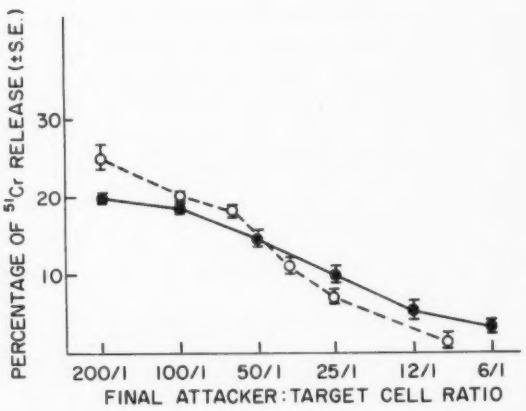

FIGURE 2.—Relationship between cytotoxic activity and decreasing ratio of attacker:target cells.
●—● ^{51}Cr release when a decreasing number of immune spleen lymphocytes is incubated with 5×10^4 labeled L1210/Ha/DIC cells.
○---○ ^{51}Cr release when an increasing number of unlabeled target cells is added to the usual attacker:labeled target cell ratio.

TABLE 2.—Extent of inhibition of immune spleen cell-mediated cytotoxicity by addition of 10^5 unlabeled cells*

Inhibitor cells	Histocompatibility relationship with DBA/2 strain	Lysis with inhibitor cells (%)/lysis without inhibitor cells (%)	Reduction in lysis (%)
L1210/Ha/DIC	Syngeneic	14.3/20.7	31†
L1210/Ha	Syngeneic	30.2/31	2
L1210/Cr	Syngeneic	19.1/21.6	11
L5178Y	Syngeneic	27.6/31	11
DBA/2 spleen cells	Syngeneic	20.1/20.7	3
CDF_1 spleen cells	Syngeneic	20.5/20.7	1
C57BL/10 spleen cells	Allogeneic	30.6/31	1
BALB/c spleen cells	Allogeneic	20.6/20.7	0
EL 4	Allogeneic	23/20.7	−11
E male G2	Allogeneic	10/10	0

*10^5 unlabeled cells were added to the incubation mixture of CDF_1 immune splenocytes plus labeled L1210/Ha/DIC cells (200:1).

†Significant difference with controls not treated with unlabeled cells ($P<0.05$ calculated on the original counts using "Student's t" test analysis). All other unlabeled cells did not elicit significant differences as compared with the controls ($P>0.05$).

cytotoxic activity of the immune spleen cells by 31%. In contrast, with all other added cells, normal or leukemic, there was little or no reduction ($\leq$11%) of the ^{51}Cr released in the incubation medium.

DISCUSSION

Appropriate chemotherapeutic treatment of a murine lymphoma in vivo can result in immunologic alteration of tumor cells. The altered cells implanted into mice compatible with the parental tumor elicit a host allograft reaction comparable to that observed with an allogeneic inoculum (10).

Spleen cells from CDF_1 mice immunized against the drug-altered leukemic line L1210/Ha/DIC elicited strong cytotoxic activity specific for L1210/Ha/DIC cells. On the other hand the immune spleen cells did not damage a series of other normal or leukemic lymphoid cells and showed limited cytotoxicity for the parental L1210 cells. Furthermore, the immune spleen cell reactivity increased with the size of the immunizing inoculum. Also, a direct relationship was observed between the lysis of labeled target cells and the ratio of immune spleen cells to target cells. As the ratio was diminished, either by reduction of attacker cells or by addition of unlabeled L1210/Ha/DIC cells, the extent of lysis of target cells was reduced.

The above experimental results confirm the immunologic basis of the cytolytic reaction and emphasize that immune spleen cells can only attack cells carrying antigen(s) to which the spleen cells were sensitized.

The specificity of the in vitro cell-mediated cytotoxicity was more definitely established by the results of the assay for inhibition of cytotoxicity. Normal or leukemic unlabeled cells of various origins added to the attacker:target cell suspension did not reduce the cytolytic activity of L1210/Ha/DIC immune spleen cells. The reduction in ^{51}Cr release was significant only when unlabeled L1210/Ha/DIC cells were added to the standard attacker:target cell suspension.

The results of this study indicate that strong antigenic expression can occur in L1210 leukemia cells after in vivo treatment with NSC-45388. The antigen(s) involved is strongly immunogenic and would appear to be in excess of any pre-existing transplantation antigen, in common with parental untreated cells.

Several mechanisms have been proposed to explain the appearance of new antigenic properties on leukemic cells (mutation, virus activation, unmasking of L1210-associated antigen[s]), but no experimental evidence has been obtained to favor a particular hypothesis.

Furthermore, in recent studies (unpublished data) no evidence for increased cellular immunosensitivity of immunologically altered cells could be detected. The cell-mediated cytotoxic reaction of immune spleen cells collected from mice sensitized with a drug-treated subline of tumor cells would therefore indicate that treatment with an antineoplastic agent can result in marked antigenic alteration of tumor cells in vivo, producing new antigenic targets or inducing higher concentrations of previously existing antigen(s), of possible significance for therapy.

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Antigenic changes of L1210 leukemia in mice treated with 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide. Cancer Res 32:1446–1450, 1972.

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Effects of 2,2′-(Methylenediimino)bis-1,3,4-thiadiazole (NSC-143019) on Tumor Growth and Immune Responses in Mice [1,2]

Takao Matsumoto,[3] Koichiro Ootsu,[3] and Yoshiyuki Okada [4]

SUMMARY

NSC-143019 was effective against L1210 leukemia, 6C3HED/OG lymphosarcoma, C1498 myeloid leukemia, Ehrlich carcinoma, Sarcoma 180, B16 melanoma, and X5563 myeloma at nontoxic doses in mice. Effects were less pronounced in MH134 and MH129p hepatomas. The compound significantly suppressed the production of serum antibody against sheep erythrocytes. Graft versus host response in adult F_1 mice was also affected by treatment with the drug. The antitumor and immunosuppressive activities of NSC-143019 were about five times higher than those of the parent compound, 2-amino-1,3,4-thiadiazole. These activities of NSC-143019, as well as its toxicity to the host, were prevented by the simultaneous administration of an equimolar amount of nicotinic acid.

[Cancer Chemother Rep 58:331–344, 1974]

The antitumor effects of a number of 2-substituted thiadiazoles have been reported by many investigators (1–10). Among them, 2-alkylamino and 2-acylamino derivatives of 2-amino-1,3,4-thiadiazole (NSC-4728) were reported to be the most effective against some leukemias in mice, although relatively less toxic (11) and somewhat diuretic (12). An antimetabolic action of these derivatives against nicotinamide was demonstrated in the growth of bacteria and tumor cells as well as in toxicity (4,13,14). However, the immunosuppressive activity of these compounds has not yet been fully explored.

We have also investigated the effects of a large number of thiadiazole compounds and have found that 2,2′-(methylenediimino)bis-1,-3,4-thiadiazole (NSC-143019) was more effective than the parent compound, NSC-4728, in retarding the growth of L1210 leukemia. The present article deals with a detailed study of the antitumor and immunosuppressive effects of NSC-143019.

MATERIALS AND METHODS

Drugs

NSC-4728 and NSC-143019 (fig 1) were synthesized by Yoshiyuki Okada (manuscript in preparation). 6-Mercaptopurine (6-MP), cyclophosphamide, methotrexate, mitomycin C, and nicotinic acid were purchased and used as positive controls. NSC-143019 and 6-MP were suspended in 0.2% gum arabic saline solution. Other compounds were dissolved in saline immediately before use.

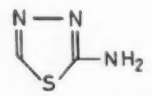
[1] Received Sept 17, 1973; revised Jan 7, 1974; accepted Jan 24, 1974.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text.

[3] Biological Research Laboratories, Central Research Division, Takeda Chemical Industries, Ltd, Osaka, Japan.

[4] Agricultural Chemical Research Laboratories, Takeda Chemical Industries, Ltd., Osaka, Japan.

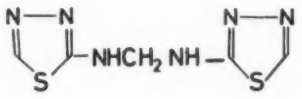
TK 4207



(2-amino-1,3,4-thiadiazole)

NSC-4728

TK 5477



N,N'-methylene-bis(2-amino-1,3,4-thiadiazole)

NSC- 143019

FIGURE 1.—Chemical structures of NSC-143019 and NSC-4728.

Animals

Specific pathogen-free C57BL/6, C3H/He, DBA/2, and hybrid (C57BL/6 × DBA/2)F_1 (BDF_1) mice of both sexes were used. Outbred JCL-ICR (ICR) mice were purchased from Japan Clea Inc., Osaka. All mice weighed 20–30 g at use.

Tumors

Ascitic L1210 leukemia [5] was maintained serially in DBA/2 mice. Sarcoma 180 (S180)[5] and B16 melanoma [5] were implanted subcutaneously (sc) by means of trocar in ICR and C57BL/6 mice respectively. Ehrlich carcinoma[5] in ascites suspension was maintained serially in ICR mice. Ascitic L1210 variants made resistant to 6-MP(L1210/6MP/R [6]), cytosine arabinoside (L1210/ara-C/R [6]), daunorubicin(L1210/DM/R [6]), and methotrexate (L1210/MTX/R [6]) were provided by Dr. A. Hoshino, Aichi Cancer Center, Nagoya, Japan, in 1972, and maintained serially in BDF_1 mice. 6C3HED lymphosarcomas sensitive (6C3HED/OG) and resistant (6C3HED/RG)[7] to L-asparaginase were provided by Dr. K. Yamada, Department of First Internal Medicine, Medical School, Nagoya University, Japan, in 1972, and implanted intraperitoneally (ip) in C3H/He mice. X5563 myeloma maintained ip in C3H/He mice was provided by Dr. M. Kitagawa, Cancer Research Institute, Osaka University, Japan, in 1971. The sources and characteristics of C1498 myeloid leukemia and MH134 and MH129p hepatomas were described elsewhere (15–17). Ascites tumor cells in an amount of 1–10 × 10^6 were inoculated either ip or sc in the respective strains of mice.

Administration of Drugs

Saline solution (0.2 ml) containing varying amounts of drugs was injected either ip or orally to mice starting 24 hours after tumor transplantation. Repeated, intermittent, or single administration schedules of drugs as outlined in the Drug Research and Development (formerly the Cancer Chemotherapy National Service Center), Division of Cancer Treatment, NCI protocols (18) were applied in this study with slight modification (19) and are shown in each table. For evaluating antitumor activity, the inhibition of tumor growth in size or the prolongation of survival time of tumor-bearing animals was compared between the drug-treated (T) and untreated (C) groups and was expressed as the percentage of growth inhibition (% inhibition in size) or by the percentage of the increase in lifespan over the controls (%ILS). Body weights of tumor-bearing animals were measured at the time of the first drug injection and usually at the last injection. Body weight loss of more than 2.0 g in the drug-treated animals was considered to be a manifestation of drug toxicity.

Micro-cytotoxicity Test in Tissue Culture

In order to evaluate the growth inhibitory activity of drugs in tissue culture, the drop-culture method was developed as follows: a droplet (0.05 ml) of cell suspension containing

[5] Provided by the National Cancer Institute (NCI), Bethesda, Md, in 1971.

[6] L1210/6MP/R, resistant to 40 mg/kg of 6-MP; L1210/MTX/R, resistant to 3 mg/kg of methotrexate; L1210/ara-C/R, resistant to 20 mg/kg of cytosine arabinoside; and L1210/DM/R, resistant to 1 mg/kg of daunorubicin.

[7] 6C3HED/RG, resistant to 250 IU/kg of L-asparaginase (from *Escherichia coli*, supplied by Worthington Biochemical Co., Freehold, NJ).

2×10^3 HeLa cells (HeLa S3) in medium 199 (M199, Grand Island Biological Co., Grand Island, NY) supplemented with 20% calf serum was placed on a sterile cover slip (18 × 18 mm) and the cover slip was put in a plastic culture dish (Falcon, No. 1008). In this manner, three dishes were settled for testing each sample. The dishes were then incubated for 18 hours at 37 C under a humidified atmosphere of 5% CO_2 and 95% air. After incubation, the supernatant medium was decanted, and 0.1 ml of control or sample medium was added to the cultures. The plating efficiency of the HeLa cells was more than 95% under these experimental conditions. Drugs were dissolved in dimethylsulfonamide (DMSO) and diluted 100–10,000 times with M199 to make the desired concentrations (final concentrations; 0.1, 1, 10, and 100 μg/ml). Control cultures were added similarly with the vehicle (1% DMSO in M199). Cell number was counted under an inverted phase microscope by means of a counting mesh (1 mm^2/area, sectioned by 10) every 24 hours after the addition of the sample solution. The living HeLa cells remained attached to the glass surface. The average number of living cells was calculated from the summation of five areas in the counting mesh located randomly on one drop-culture and was plotted as the relative cell number in the figure.

Preparation of Antigen

Sheep erythrocytes (SRBC) kept in Alsever's solution were obtained from Shiihashi Co., Tokyo, Japan. The cells (SRBC) were aseptically washed three times in sterile saline and adjusted to make a suspension containing 5×10^8 cells/ml in the same sterile diluent. For serologic testing, the concentration of SRBC was adjusted to 2% for the microhemagglutinin reaction.

Immunization

BDF_1 and C3H/He mice were immunized with an intravenous (iv) injection of 0.2 ml of a 5×10^8 SRBC suspension. Mice were bled from the heart 5 days after immunization, and the sera separated were stored at −20 C until use after heat-inactivation at 56 C for 30 minutes.

Hemagglutination

Microhemagglutination was performed in the microtiter system (Tominaga Co., Tokyo, Japan) by the standard method. The higher dilution showing the apparent agglutination of the SRBC was taken as the final antibody titer. Antibody titers less than one fourth of the level in the untreated group were regarded as demonstrating positive immunosuppression in the drug-treated group (16,19,20).

Hemolytic Plaque-Forming Cell (PFC) Assay

Spleen cells releasing 19S hemolysin 4 days after immunization of C3H/He mice with SRBC were enumerated on petri dishes according to the method of Jerne and Nordin (21). Spleens were removed aseptically from mice and teased in cold, sterile M199 to obtain a single-cell suspension. One spleen was dispersed in 1 ml of M199 and diluted appropriately. Five mice were used for each experimental group, and duplicate plates were prepared for each spleen. Thus, each number reported in the table is the mean of the plaque counts on ten plates (± standard deviation) expressed as the number of plaques per spleen or 1×10^8 spleen cells.

Cell Counts

Tumor cells were counted in a hemocytometer using Turk's solution. Spleen cell counts were performed with a Coulter Model B cell counter (Coulter Electronics, Hialeah, Fla), with the lower threshold set to exclude erythrocytes and cell debris. The viability of cells was determined by the trypan blue dye exclusion method.

Graft Versus Host (GVH) Reaction

For the production of GVH responses in adults (22,23), BDF_1 females, 6–8 weeks old, were used as recipients of spleen cell suspensions from C57BL/6 donors. For the transfer of spleen cells, pooled suspensions of spleen cells from donor C57BL/6 mice, separated by teasing and filtering through steel mesh and washed in Hank's balanced salt solution (HBSS) three times by centrifuging (600 rpm), were adjusted to contain 2×10^8 cells/ml in HBSS; 0.2 ml of the spleen cell suspension was injected iv into BDF_1 recipient mice. Ten days after the transfer of spleen cells, the mice were

killed and the body and spleen weights were measured. The results were expressed as ratios of milligrams of spleen weight to 1 g of body weight (spleen index). Average numbers of spleen indices from five mice per group (± standard error) are indicated in the table. Drugs were administered to the recipients ip for 5 consecutive days starting from 48 hours after the transfer of spleen cells.

RESULTS

Toxicity of NSC-143019 to Normal and Tumor-Bearing Mice

NSC-143019 given in single ip doses of 1500, 1750, and 2000 mg/kg resulted in the death of all C3H/He mice from 2 to 6 days after treatment (fig 2). The doses of 1000 and 1250 mg/kg resulted in 20% and 40% mortality of animals respectively, while simultaneous oral administration of 500 mg/kg of nicotinic acid decreased the mortality of the mice treated with 1500 mg/kg of NSC-143019 from 100% to 20% (fig 2).

Toxicity of NSC-143019 and NSC-4728 was compared in normal C57BL/6 mice using five mice per group. Five daily ip injections of 100 and 200 mg/kg of NSC-143019 produced no deaths in the mice and no body weight loss. The higher doses of NSC-143019 resulted in 40% mortality at 300 mg/kg, 80% mortality at 400 mg/kg, and 100% mortality at 500 mg/kg. The same procedures using NSC-4728 at doses of 600, 800, 1000, and 1200 mg/kg resulted in no deaths and produced 100% mortality at 1400 mg/kg. Five daily oral doses of 200 and 400 mg/kg of NSC-143019 and 800 and 1200 mg/kg of NSC-4728 were tolerable in C57BL/6 mice. However, 1200 mg/kg of NSC-143019 and 2000 mg/kg of NSC-4728 in the same regimen produced 100% mortality of the mice.

Groups of C3H/He male mice received sc implants of 5×10^5 tumor cells of X5563 myeloma and ip injections of varying doses of NSC-143019 for 10 consecutive days, starting 24 hours after tumor implantation. Other groups of C3H/He mice with no tumor implantation received injections of NSC-143019 according to the same schedule as above and

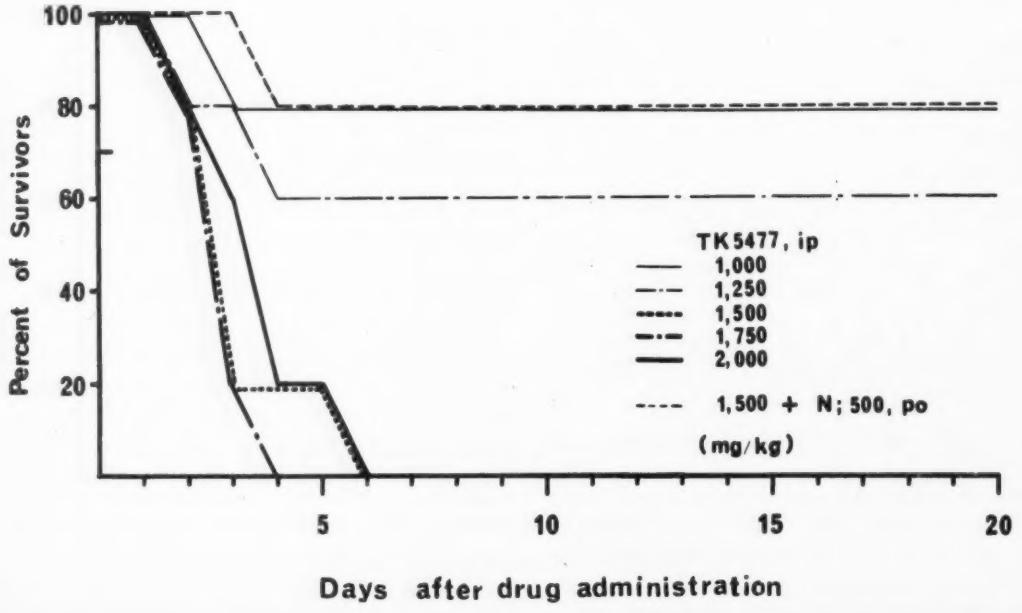


FIGURE 2.—Percent of C3H/He male mice surviving after a single ip administration of various doses of NSC-143019 (TK5477) with or without the simultaneous injection of nicotinic acid. N = nicotinic acid, given orally.

served as controls. In normal C3H/He mice the daily doses of 75, 100, and 150 mg/kg of NSC-143019 produced neither death nor significant body weight loss. On the other hand, in tumor-implanted C3H/He mice, NSC-143019 at a dose of 100 mg/kg produced 40% toxic deaths, and at 150 mg/kg all animals were dead due to toxicity. Lower doses of 75 and 50 mg/kg of NSC-143019 resulted in no toxic deaths of the tumor-bearing animals and thereby elongated the survival time over that of the controls (table 1). Combined iv administration of 100 mg/kg of nicotinic acid daily for 10 days significantly decreased the toxicity of the tumor-implanted mice treated ip with 100 mg/kg of NSC-143019. Similar administration of nicotinic acid to the animals treated with NSC-143019 at a dose of 200 mg/kg reduced the toxic deaths from 100% to 40% and consequently elongated the survival time of the tumor-bearing animals (table 1).

Antitumor Activity

Effects on L1210 leukemia.—One million L1210 cells were inoculated ip in groups of five BDF_1 mice. The single ip doses of 100 and 250 mg/kg of NSC-143019 on the first day after the tumor implantation produced ILSs of 53% and 80% respectively. The same procedures using 500 and 1000 mg/kg resulted in the death of the animals within 5 days, probably due to the toxicity of NSC-143019. Daily ip doses of 10–80 mg/kg of NSC-143019 for 5 days prolonged the survival time to a relatively uniform extent and produced a negligible body weight loss in the tumor-bearing animals (table 2). Injection of 20 mg/kg of NSC-143019 provided a 44% ILS. The effects of NSC-4728 given according to the same schedules used for NSC-143019 were variable, and only the dose of 200 mg/kg showed a positive increase in survival time. Five daily oral doses of 50, 100, and 200 mg/kg of NSC-143019 produced a considerably uniform prolongation of the survival time with negligible body weight loss, and the dose of 400 mg/kg was toxic. NSC-4728 was also effective orally in the same dose ranges used for NSC-143019 (table 2). For the determination of the most suitable treatment regimen, groups of five to ten BDF_1 mice were inoculated ip with 1×10^6 L1210 cells and received repeated oral administrations of 50 mg/kg of NSC-143019 or cyclophosphamide at the various intervals indicated in figure 3: *A* = four daily treatments successively; *B* = four daily treatments successively, followed by one more on the seventh day; *C* = four daily treatments followed by three treatments every third day starting on the seventh day; *D* = four daily treatments followed by four treatments every second day; and *E* = repetition of four daily treatments from 1, 7, and 13 days after tumor implantation. Compared to the other schedules, NSC-143019 significantly prolonged the survival time when used according to schedule C (fig 3).

Effects on the drug-resistant sublines of L1210 and other ascites tumors.—BDF_1 mice bearing L1210/6MP/R or L1210/ara-C/R responded well to the repeated ip doses of 25 or 50 mg/kg of NSC-143019 given daily for 5 days. However, no antitumor effect from the same procedure using NSC-143019 was demonstrated in the mice bearing L1210/MTX/R or L1210/DM/R. The daily doses of 10 and 20 mg/kg of

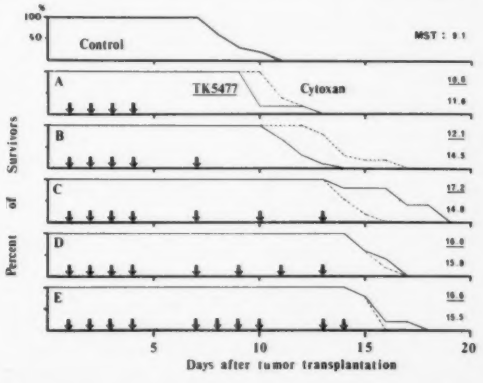


FIGURE 3.—Prolongation of survival time of L1210-bearing BDF_1 mice by oral treatment with NSC-143019 (TK5477) or cyclophosphamide (Cytoxan). One million L1210 cells were implanted ip and drugs were administered orally at a dose of 50 mg/kg at the various intervals indicated. Solid lines in *A*, *B*, *C*, *D*, and *E* indicate survival curve of NSC-143019-treated tumor-bearing mice; broken lines indicate survival curve for cyclophosphamide. Five mice per treatment group and 10 mice per control group. Arrows indicate the injections of NSC-143019 and cyclophosphamide. MST = mean survival time (underlined for NSC-143019).

TABLE 1.—Subacute toxicity of NSC-143019 in normal and tumor-bearing C3H/He mice and effect of nicotinic acid

Tumor	Dose of NSC-143019* (mg/kg/day, ip)	Without nicotinic acid					With nicotinic acid¶				
		No. of toxic deaths/ total treated	MST (days) of animals with toxic deaths†	No. of tumor deaths/ total treated	MST (days) of animals with tumor deaths‡	Body weight change§ (g)	No. of toxic deaths/ total treated	MST (days) of animals with toxic deaths†	No. of tumor deaths/ total treated	MST (days) of animals with tumor deaths‡	Body weight change§ (g)
None	75	0/5				0.2	0/5				−1.0
	100	0/5				−0.2					
	150	0/5				0	0/5				−1.4
	Untreated**	0/5				−0.2	0/5				−0.4
X5563 myeloma††	50	0/5		5/5	23.8	2.4					
	75	0/5		5/5	24.4	1.5					
	100	2/5	8.5	3/5	24.7	0.7	0/5		5/5	22.4	3.5
	150	5/5	7.8								
	200	5/5	7.8				2/5	5.5	3/5	25.7	2.0
	Untreated**	0/5		5/5	17.4	6.7	0/5		5/5	17.6	7.7

*Varying doses of NSC-143019 were injected daily for 10 days.

†Calculated from lifespans of animals that died of toxicity.

‡Calculated from lifespans of animals that died of tumor.

§Body weight was measured at the time of the 1st (1 day after tumor implantation) and last (10 days after tumor implantation) drug administration, and the changes were expressed as an increase or a decrease in body weight.

¶Nicotinic acid at a dose of 100 mg/kg was injected simultaneously with NSC-143019 for 10 days iv.

**Dose of 0.2 ml of physiologic saline was injected ip daily for 10 days.

††X5563 myeloma cells (5×10^5) were inoculated sc 1 day before the start of drug administration.

TABLE 2.—Effects of repeated ip or oral doses of NSC-143019 or NSC-4728 on the survival time of animals bearing L1210

Drugs	Dosage* (mg/kg/day × 5)	Route of injection	No. of mice	MST (days) (treated/control)	%ILS	Average body weight change (g), Days 1–5 (treated/control)
NSC-143019	10	ip	5	10.4/9.0	16	0.6/1.0
	20		5	13.0/9.0	44	0.4/1.0
	30		5	11.8/9.0	31	0.8/1.0
	40		5	12.2/9.0	36	0.4/1.0
	50		5	12.2/9.0	36	0.4/1.0
	60		5	13.4/9.0	49	0.6/1.0
	80		5	14.0/9.0	56	0.8/1.0
NSC-4728	50	ip	5	10.8/9.0	20	0.4/1.0
	100		5	11.2/9.0	24	0.6/1.0
	200		5	12.6/9.0	40	0/1.0
	300		5	9.2/9.0	2	−0.8/1.0
	500		5	9.6/9.0	7	−1.6/1.0
Cyclophosphamide	50	ip	5	12.0/9.0	33	0.2/1.0
	100		5	13.2/9.0	47	0.4/1.0
NSC-143019	50	oral	10†	12.3/9.5	29	−0.2/1.1
	100		10†	12.9/9.5	36	0/1.1
	200		10†	13.6/9.5	43	0/1.1
	400		5	9.6/9.0	7	−1.4/1.0
NSC-4728	50	oral	5	13.0/10.0	30	0.4/1.2
	100		5	14.4/10.0	44	0.2/1.2
	200		5	14.6/10.0	46	−0.6/1.2
	300		5	11.4/10.0	14	−0.6/1.2
	400		10†	9.4/9.5	0	−1.0/1.1
	800		5	7.2/9.0	0	−2.4/1.0
Cyclophosphamide	50	oral	10†	14.1/9.5	48	−0.7/1.1
	100		5	15.8/9.0	76	−1.6/1.0

*One million L1210 cells were transplanted ip and drugs were administered daily for 5 days starting 24 hours after tumor transplantation.

†Results of 2 experiments.

NSC-143019 were highly effective in mice with 6C3HED/OG lymphosarcoma sensitive to L-asparaginase. In contrast, the mice bearing 6C3HED/RG resistant to the enzyme were insensitive to the dose of 25 mg/kg of NSC-143019. Twenty-five and 50 mg/kg of NSC-143019 were ineffective in mice with MH134 and MH129p hepatomas (table 3).

Effects on solid tumors.—Tumor cells (1 or 10 million) or tumor pieces (2 mm^3 in size) were implanted sc in the sensitive strain of mice. Using groups of five mice, daily ip or oral administration was started 24 hours after the implantation of the tumors. Daily ip doses of 50 mg/kg of NSC-143019 for 5 days in the mice implanted sc with S180, 6C3HED/OG, or B16 melanoma inhibited the tumor growth by nearly 50% compared to the controls (table 4). The solid hepatomas, MH134 and MH129p, were insensitive to the dose of NSC-143019. The daily oral doses of 50 and 100 mg/kg of NSC-143019 given for 5 days depressed (dose-dependently) the tumor size in the mice implanted with L1210. Similarly, Ehrlich carcinoma transplanted sc in ICR mice was also sensitive to the daily oral dose of 100 mg/kg of NSC-143019. MH134 and MH129p hepatomas were less sensitive to the oral dose of 100 mg/kg of NSC-143019 (table 4).

TABLE 3.—Effects of NSC-143019 on various kinds of ascites tumors in mice*

Tumor†	Dosage (mg/kg/day × 5, ip)	MST (days) (treated/control)	%ILS	Average body weight change (g), Days 1–5 (treated/control)
L1210/6MP/R	25	12.7/9.3	37	1.3/2.0
L1210/ara-C/R	25	10.7/8.2	30	1.7/4.2
	50	11.5/8.2	40	1.3/4.2
L1210/MTX/R	25	9.2/10.2	0	–1.8/–1.6
	50	9.0/10.2	0	–2.8/–1.6
L1210/DM/R	25	8.8/8.6	2	–1.2/0.8
	50	8.6/8.6	0	–1.6/0.8
6C3HED/OG	10	30.0/14.0	114	–1.0/–0.2
	20	24.2/14.0	73	–1.4/–0.2
6C3HED/RG	25	8.8/11.0	0	–1.8/2.4
MH134	25	11.2/15.8	0	–2.8/1.2
MH129p	25	26.4/22.6	17	–0.4/0.6
	50	19.7/22.6	0	0.4/0.6

*One million L1210 cells or 1×10^7 cells of other kinds of tumors were transplanted ip, and drugs were administered ip daily for 5 days starting 24 hours after tumor transplantation. Five mice per group.

†For explanation of abbreviations *see* Materials and Methods.

TABLE 4.—Effects of NSC-143019 on the growth of sc transplanted tumors in mice*

Tumor	Dosage† (mg/kg/day)	Route	Mean tumor size: Day of assessment	Mean tumor size: mm (treated/control)	Inhibition (%)	Average body weight change (g), Days 1–5 (treated/control)
S180	25 × 5	ip	16	10.5/19.3	46	–0.8/3.2
	50 × 5	ip	16	10.0/19.3	48	–2.7/3.2
B16	10 × 5	ip	15	17.5/21.5	19	–0.2/0.1
	25 × 5	ip	15	17.0/21.5	21	–0.6/0.1
	50 × 5	ip	15	11.2/21.5	48	–0.8/0.1
6C3HED/OG	25 × 10	ip	11	10.7/17.2	38	2.3/2.6
	50 × 10	ip	11	8.1/17.2	53	–0.3/2.6
MH134	25 × 10	ip	12	15.8/17.6	10	1.2/1.3
	50 × 10	ip	12	13.1/17.6	26	0.8/1.3
MH129p	25 × 10	ip	12	14.9/16.1	7	0.8/1.1
	50 × 10	ip	12	13.2/16.1	18	0.2/1.1
L1210	50 × 5	oral	7	7.1/10.5	32	1.8/2.2
	100 × 5	oral	7	4.6/10.5	56	1.2/2.2
Ehrlich	100 × 5	oral	9	7.3/13.3	45	–2.9/1.0
MH134	100 × 10	oral	12	11.9/17.6	32	1.2/1.3
MH129p	100 × 10	oral	12	12.7/16.1	21	0.4/1.1

*B16 melanoma and S180 were transplanted sc using trocar pieces (2 mm^3 in size), and 1 million L1210 cells and 10 million of other kinds of ascitic tumor cells were transplanted sc. Five mice per group.

†Drug was injected ip or orally daily for 5 or 10 days starting 24 hours after tumor implantation.

Antagonism of antitumor effect by nicotinic acid.—Repeated ip doses of 50 mg/kg of NSC-143019, starting 24 hours after sc implantation of L1210, prolonged the survival time of the mice and reduced the tumor sizes. In such animals, the simultaneous administration of nicotinic acid by mixing in drinking water (1 mg/ml) almost completely prevented the antitumor effects of NSC-143019 (fig 4). Similar antagonism between NSC-143019 and nicotinic acid was demonstrated in the mice inoculated sc with C1498 leukemic cells.

Antagonism in tissue culture.—The growth of HeLa S3 cells was significantly depressed (dose-dependently) at concentrations of 1.0 and 100 μg/ml of NSC-143019 or NSC-4728 (fig 5). The simultaneous addition of equimolar amounts of nicotinic acid to the culture medium almost completely reversed the growth-inhibitory activity of the two drugs.

Cytotoxic Activity

The ip doses of NSC-143019 produced a decrease in the number of mitotic cells until about 15 hours after the administration when compared to those of the control animals, and thereafter the number of mitotic cells increased in the treated animals (data not shown). Destructive figures of tumor cells appeared less prominently from 18 to 24 hours after administration of the single dose of 50 or 100 mg/kg of NSC-143019. The tumor cells undergoing destructive changes were characterized by the enlargement of the cell body with many cytoplasmic granules and vacuoles. These features were somewhat different from those which appeared in the tumor cells treated with mitomycin C and cytosine arabinoside (20). The striking mitotic or chromosomal abnormalities, which were typically observed in the tumor cells treated with cytosine arabinoside or mitomycin C, were rarely found in the tumor cells of the mice treated with NSC-143019.

Immunosuppressive Effects

Effect of hemagglutinin production.—As shown in figure 6, ip treatment with NSC-143019 or NSC-4728 daily for 4 days starting 24 hours after the antigen injection resulted in a significant decrease in antibody titers occurring 5 days after immunization of BDF_1 mice with SRBC. NSC-143019 at a dose of 50 mg/kg and NSC-4728 at a dose of 300 mg/kg completely suppressed the antibody production, and the degree of inhibition was reduced by decreasing the amount of the compounds administered (fig 6). A more marked depression of the production of hemagglutinin was obtained when NSC-143019 or NSC-4728 was given 2 days after the antigen. Further increase in the interval between the antigen and the compound administration resulted in the proportional decrease in depressive activity. When the serum antibody titers were evaluated 9 days after the antigen was given, both drugs given even 2 days after the antigen were no longer effective. In C3H/He mice immunized with 1×10^8 SRBC, the combined sc administration of nicotinic acid at doses of 1, 10, and 50 mg/kg antagonized (dose-dependently) the suppressive effect of 50 mg/kg of NSC-143019 (fig 7).

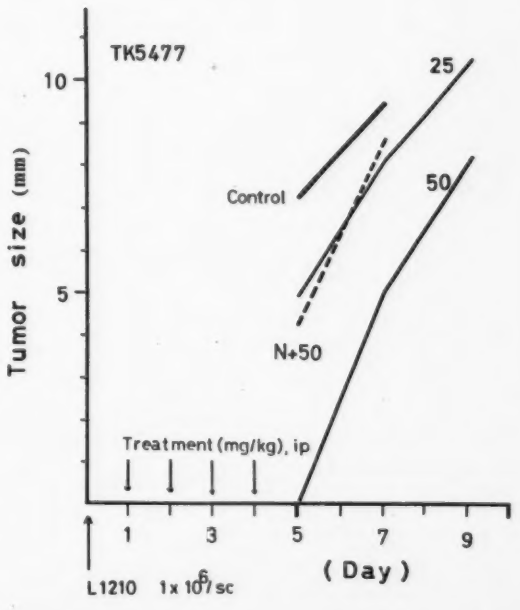


FIGURE 4.—Effect of NSC-143019 (TK5477) on the growth of L1210 leukemia implanted sc and the reversal by nicotinic acid. N + 50 = nicotinic acid mixed in drinking water (1 mg/ml) and administered for 4 days starting on the day 50 mg/kg of NSC-143019 is given.

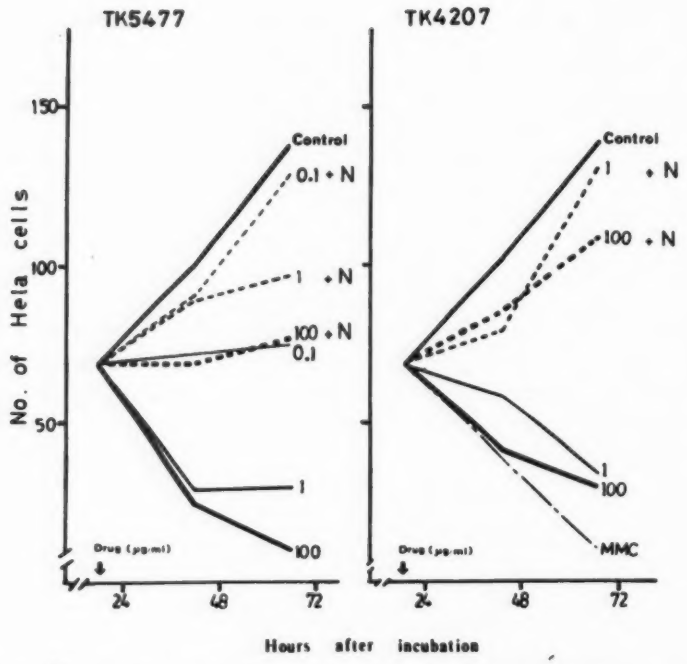


FIGURE 5.—Effects of NSC-143019 (TK5477) and NSC-4728 (TK4207) on the growth of HeLa cells and the reversal by nicotinic acid in vitro. N = nicotinic acid, given in the same quantity as NSC-143019 or NSC-4728. M199 contained 0.25 μg/ml of nicotinic acid and nicotinamide as constituents. The additional administration of nicotinic acid to the medium at a concentration of 100 μg/ml had no influence on the growth of HeLa cells. MMC = mitomycin C, 10 μg/ml. Numbers headed on each line indicate the concentrations of drug (μg/ml) added.

TABLE 5.—Effect of a single injection of NSC-143019 on the primary antibody-forming cell (PFC) responses in spleens of mice immunized with sheep erythrocytes (SRBC)

Antigen*	NSC-143019 (200 mg/kg, ip) given on the indicated day after antigenic stimulation	PFC response on Day 4†	
		PFC/spleen	PFC/10^6 nucleated spleen cells
SRBC	None	620,000 ± 99,454	432,190 ± 82,007
None	None	500 ± 76	396 ± 76
SRBC	1	123,480 ± 33,895	77,788 ± 20,211
SRBC	2	26,000 ± 8071	22,799 ± 7313
SRBC	3	156,520 ± 24,207	139,735 ± 19,221

*C3H/He female mice received iv injections of 1×10^8 SRBC.

†No. of PFCs was assessed 4 days after immunization. Averages of 5 mice ± standard deviation.

TABLE 6.—Effects of NSC-143019 and 6-MP on the GVH reaction in adult F_1 mice*

Transfer of spleen cells	Treatment† (mg/kg/day × 5)	Spleen index (mg/g body weight) (mean of 5 mice ± SE)	Inhibition‡ (%)
No	None	3.36 ± 0.26	—
Yes	None	9.59 ± 0.59	—
Yes	NSC-143019, 10	5.27 ± 0.17	69
Yes	NSC-143019, 20	6.07 ± 0.50	57
Yes	NSC-143019, 40	6.68 ± 0.39	47
Yes	NSC-143019, 40 + nicotinic acid, 10§	6.83 ± 0.92	44
Yes	6-MP, 20	5.17 ± 0.14	71
Yes	6-MP, 40	3.99 ± 0.30	90

*(C57BL/6 × DBA/2)F_1 mice received iv injections of C57BL/6 spleen cells (4×10^7) and their spleens were sampled 10 days later.

†Drugs were injected ip for 5 consecutive days starting 48 hours after cell transfer.

‡Inhibition $= \left[1 - \frac{\text{spleen index of treated mice—spleen index of normal mice}}{\text{spleen index of untreated mice—spleen index of normal mice}}\right] \times 100.$

§Nicotinic acid given iv.

Effect on PFC response.—Groups of female C3H/He mice were immunized iv with 1×10^8 SRBC and treated ip with a single dose of 200 mg/kg of NSC-143019 on the first, second, or third day after the antigen. The PFC responses were measured 4 days after immunization. The most marked depression of the PFC responses was observed in the mice treated with NSC-143019 2 days after antigenic stimulus (table 5).

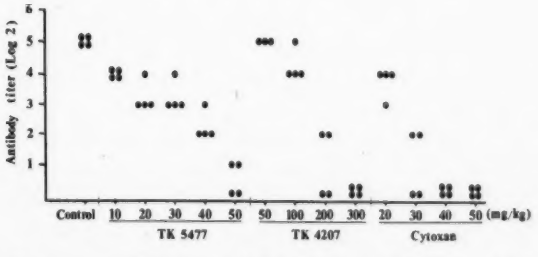


FIGURE 6.—Effects of NSC-143019 (TK5477), NSC-4728 (TK4207), and cyclophosphamide (Cytoxan) on the antibody production in BDF_1 female mice. BDF_1 mice were immunized iv with 1×10^8 SRBC. Drugs were injected ip for 4 consecutive days starting 24 hours after immunization. Hemagglutinin titer of each mouse immunized with SRBC was measured 5 days after the antigen. Each point indicates 1 mouse.

Effect on GVH reaction.—Forty million spleen cells taken from donor C57BL/6 mice were injected iv into groups of BDF_1 mice, resulting in a significant increase in the weight of the spleen 10 days later. The daily ip doses of 10, 20, and 40 mg/kg of NSC-143019 for

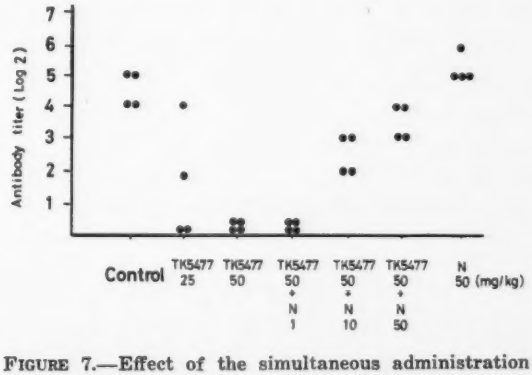


FIGURE 7.—Effect of the simultaneous administration of nicotinic acid on the immunosuppressive activity of NSC-143019 (TK5477) in C3H/He female mice. Mice were immunized iv with 1×10^8 SRBC. NSC-143019 was injected ip and nicotinic acid (N) was injected sc at the time of NSC-143019 treatment. Hemagglutinin titer of each mouse (each point) immunized with SRBC was measured 5 days after the antigen.

5 days, starting 48 hours after the spleen cell transfer, uniformly depressed the increase in spleen index. The same procedure with 20 and 40 mg/kg of 6-MP depressed the spleen index somewhat more markedly. The combination of 10 mg/kg of nicotinic acid given daily through an iv route along with NSC-143019 did not significantly affect the depression of the spleen index by NSC-143019 (table 6).

DISCUSSION

NSC-143019 exhibited considerable antitumor activity in mice (tables 3 and 4). The minimum effective dose of the drug given by the ip route, for increasing the survival time of mice bearing ascitic L1210, was approximately 20 mg/kg, whereas that of NSC-4728 was about 100 mg/kg. The relative effectiveness of NSC-143019 against L1210 was then approximately five times greater than that of NSC-4728. It was interesting that NSC-143019 was markedly inhibitory against L1210 leukemias resistant to 6-MP and cytosine arabinoside but was not inhibitory against those resistant to methotrexate and daunorubicin or against lymphosarcoma resistant to L-asparaginase (6C3HED/RG). Cytologic studies have shown that NSC-143019 affects mainly the tumor cells in the logarithmic phase of proliferation without showing prominent cytopathic effects on the tumor cells, and the recovery of growth in the tumor cells is rather rapid if no increased administration of NSC-143019 is given. These facts imply that the drug is likely to have an antimetabolic action on the proliferating tumor cells.

NSC-143019 was relatively less toxic to normal mice (table 2, fig 1). The repeated administrations of the drug at 150 mg/kg for 10 days did not result in any acute deaths of C3H/He male mice. However, the same dose was intolerable to the mice bearing X5563 myeloma. This suggests a difference in the metabolic patterns of NSC-143019 between normal and tumor-bearing animals. The toxic manifestations that appeared in mice after treatment with large doses of the drug were in close similarity to those of NSC-4728 as reported by Humphrey et al (*4*). In addition, autopsy findings have indicated that repeated administrations of NSC-143019 at daily ip doses of 1 mg/kg for 3 months result in marked lipophilia in the bone marrow, appearance of normoblasts in the spleen, and atrophy of the thymus in rats.[8] However, the subacute toxicity of NSC-143019 was reported to be weaker in mice than in rats.[8] Such a difference observed between mice and rats in sensitivity to NSC-143019 may be caused by a rapid rate of accumulation of toxic metabolites in rats resulting abruptly in the death of the animals.

Immunosuppression of thiadiazole derivatives has not been reported yet. NSC-143019 exhibited a marked effect in suppressing serum antibody production in mice against SRBC. NSC-4728 was also immunosuppressive to a similar extent. Furthermore, NSC-143019 interfered with the GVH response elicited in adult (C57BL/6 × DBA/2)F_1 mice by administering the parental C57BL/6 spleen cells (table 6). From these findings, it was reasonable to consider that NSC-143019 exerted its effects especially on the function of tissues associated with immune responses. This speculation is supported by histopathologic findings indicating destruction of hematopoietic and thymus tissues in the mice that died from large doses of NSC-143019.[8] The greatest suppression of the serum hemagglutinin and PFC responses in mice occurred when NSC-143019 was administered 2 days after antigenic stimulation. These facts imply that NSC-143019 may damage the immunocompetent cells as well as the tumor cells at the logarithmic phase of proliferation. It is therefore probable that the suppressed immune response is attributed to the decreased number of potent immunocompetent cells, and that antitumor and immunosuppressive activities of NSC-143019 are working with a similar mode of action.

The antitumor and immunosuppressive activities of NSC-143019, as well as the toxicity to the hosts, could be completely abolished by the simultaneous administration of an equimolar amount of nicotinic acid. The mode of action of thiadiazole derivatives has been investigated and discussed with relevance to this nicotinamide antagonism (4–6,13,24,25). Teratogenic activity of NSC-143019 as well as NSC-4728

[8] Miyaji T. Personal communication.

was also protected by nicotinamide.[9] The extent of protection by nicotinic acid of the antitumor and immunosuppressive activities of NSC-143019 appeared to be of the same order as that of NSC-4728. This finding suggests that the injurious action of NSC-143019 to tumor cells or lymphoid cells may be caused by the same or similar metabolic phenomena as in NSC-4728.

LIST OF COMPOUNDS

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151)

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-; MTX; amethopterin

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

Nicotinic acid: NSC-169454; CAS reg. No. 59-67-6; 3-pyridinecarboxylic acid; Niacin

NSC-4728: CAS reg. No. 4005-51-0; 1,3,4-thiadiazole, 2-amino-; TK-4207

NSC-143019: CAS reg. No. 26907-37-9; 1,3,4-thiadiazole, 2,2'-(methylenediimino)bis-; TK-5477

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Antitumor Activity and Mode of Action of 2-Thio-6-azauridine (NSC-146268) on L1210 Leukemia [1,2,3]

L. H. Li, G. L. Neil, T. E. Moxley, and E. J. Olin [4,5,6]

SUMMARY

This study attempts to evaluate the antitumor effect of 2-thio-6-azauridine against L1210 leukemia and to elucidate its mechanism of action. This drug has a cytotoxic effect on L1210 leukemic cells growing in culture with 50% and 90% inhibition dose values of 0.06–0.11 and 0.23–0.46 μg/ml, respectively. It is also active against L1210 leukemia in vivo. At 0.08 mM/kg/day for 9 days, approximately a 30% increase in lifespan was observed. The cytotoxic effects of this drug on L1210 leukemic cells both in vitro and in vivo approach those achieved with 6-azauridine, which was used as a reference compound in this study. Over a 4-hour period of incubation, 2-thio-6-azauridine significantly inhibited orotic acid transformation and subsequent incorporation but did not inhibit the incorporation of thymidine, uridine, or leucine into macromolecules. This inhibition was almost completely prevented by the addition of uridine, and was partially prevented by cytidine. Neither deoxyuridine, deoxycytidine, adenosine, nor guanosine had any effect. 2-Thio-6-azauridine was found to inhibit the transformation of orotic acid to phosphorylated products of uridine by inhibiting orotidylic acid decarboxylase. Thus, all evidence showed that 2-thio-6-azauridine, after conversion to the 5′-phosphate, interrupted the de novo synthesis of pyrimidine nucleotides by specific inhibition of the enzyme orotidylic acid decarboxylase.

[Cancer Chemother Rep 58:345–352, 1974]

[1] Received Sept 21, 1973; revised Jan 15, 1974; accepted Jan 24, 1974.

[2] Supported in part by contract PH43-68-1023 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] 2-Thio-6-azauridine: CAS reg. No. 27089-56-1; *as*-triazine-3,5(2*H*, 4*H*)-dione, 2-β-D-ribofuranosyl 3-thio-.

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[6] The authors wish to thank Dr. J. H. Hunter and Mr. H. Skulnick for supplying the compounds 2-thio-6-azauridine and 2-thio-6-azauracil; Mrs. J. W. Culp for her skillful technical assistance; and Ms. E. L. Clark and Ms. M. J. Campbell for their help in the preparation of this manuscript.

2-Thio-6-azauridine and 2-thio-6-azauracil [7] were chemically synthesized by Dr. J. H. Hunter in the Upjohn research laboratories. We are reporting the antitumor effect and a proposed mechanism of action of 2-thio-6-azauridine, both in vitro and in vivo, on the experimental L1210 tumor system. 6-Azauridine [8] was used in these studies for the purpose of comparison.

MATERIALS AND METHODS

Chemicals and medium.—Radioactive thymi-

[7] 2-Thio-6-azauracil: NSC-146269; CAS reg. No. 626-08-4; *as*-triazine-3,5(2*H*,4*H*)-dione, 3-thio-.

[8] 6-Azauridine: NSC-32074; *as*-triazine-3,5(2*H*,4*H*)-dione, 2-β-D-ribofuranosyl-.

TABLE 1.—Effect of 2-thio-6-azauridine on the macromolecular synthesis of L1210 cells in culture*

2-Thio-6-azauridine concentration (μg/ml)	Thymidine-^{3}H		Uridine-^{3}H		Orotic acid-^{3}H		Leucine-1-^{14}C	
	SA†	%‡	SA	%	SA	%	SA	%
0	875	100.0	550	100.0	312	100.0	16.1	100.0
2	747	85.4	557	101.3	118	37.8	15.5	96.3
10	753	86.1	522	94.9	44	14.1	13.2	82.0
50	914	104.5	519	94.4	28	9.0	22.0	136.6

*L1210 cells (about 5×10^5 cells/ml) were incubated at 37 C simultaneously with 2-thio-6-azauridine at different concentrations and a given labeled metabolite (thymidine-^{3}H or uridine-^{3}H [25 millicuries/12 mμM/ml of cells]; leucine-1-^{14}C [0.5 millicuries/200 mμM/ml]; and orotic acid-^{3}H [2.5 millicuries/18 mμM/ml]) for up to 4 hrs (within the initial rate of incorporation and the standard deviation of 5%). The DNA and RNA fractions were obtained by extracting the washed cell pellet twice with 1 ml of 0.5N perchloric acid (PCA) for 20 min at 70 C and the protein fraction was obtained by digesting the washed cell pellet with 2 ml of 1N NaOH at 37 C overnight. The extracts were then used for counting radioactivities and determining the nucleic acid and protein contents.

†SA = specific activity (cpm/μg of DNA, RNA, or protein) for a period of 2-hr incorporation.

‡% = Percent of control calculated from the specific activity of incorporation.

dine, uridine, orotic acid, orotidine-5′-phosphate, and leucine were obtained from New England Nuclear Corporation (Boston, Mass). Tritiated 6-azauracil[9] was purchased from Schwartz/Mann (Orangeburg, NY). RPMI 1634 medium and supplements were supplied by Grand Island Biological Company (Grand Island, NY).

L1210 cell-growth study.—L1210 cells were maintained in basal medium developed by Moore et al (1); the details of the procedure were described in the legends. To determine ID50 and ID90 values, 5 ml of L1210 cells (5×10^3 cells/ml) was incubated with the drug at 37 C for 3 days, and cell growth was determined (2).

Antitumor activity.—The methods used in studying the effects of agents on the survival of L1210 leukemic mice were similar to those described by Drug Research and Development (formerly the Cancer Chemotherapy National Service Center), DCT, NCI (3). A 25% increase in lifespan (ILS) is considered to be significant antitumor activity.

Macromolecular synthesis.—The details of the procedure are described in table 1. RNA content was determined by the orcinol method (4); DNA content was determined by Burton's modification of the diphenylamine method (5); protein content was determined according to the procedure of Lowry et al (6).

Enzyme assays.—A crude cell-free extract of either erythrocyte-removed ascitic cells or L1210 cells in culture was used as the source of enzymes. The isolation procedure was described elsewhere (7). Uridine kinase was determined according to the procedure of Chu and Fischer (8). The reaction was terminated by heating for 2 minutes in a boiling water bath, and the phosphorylated derivatives were separated by thin-layer chromatography (silica gel) via the solvent system of methyl ethyl ketone:acetone:water in a 7:2:1 ratio. Orotidine-5-phosphate (OMP) decarboxylase activity was determined according to the procedure of Holmes (9). The reaction mixture was, however, modified as described in table 2, and the reaction was carried out at 37 C instead of 25 C.

RESULTS

Antitumor activity and cytotoxicity.—The cytotoxicity of four drugs against L1210 cells in culture is shown in table 3. 2-Thio-6-azauridine is somewhat less cytotoxic than 6-azauridine. 6-Azauracil and 2-thio-6-azauracil, the bases, are much less active. The effect of 2-thio-6-azauridine on the survival of L1210 leukemic mice is summarized in table 4. Daily intraperitoneal (ip) doses of 2-thio-6-azauri-

[9] 6-Azauracil: NSC-3425; CAS reg. No. 461-89-2; *as*-triazine-3,5(2*H*,4*H*)-dione.

TABLE 2.—Effect of 2-thio-6-azauridine on OMP decarboxylase of L1210 cells in culture and in vivo*

2-Thio-6-azauridine concentration (μg/ml)	L1210 cells in culture		L1210 cells in vivo	
	SA†	Inhibition (%)	SA	Inhibition (%)
0 (control)	12.11 ± 1.05	0	5.51 ± 0.80	0
10	9.76 ± 3.32	19.4	2.55 ± 1.91	53.8
25	5.12 ± 0.48	57.7	1.14 ± 0.07	79.2
50	0.12 ± 0.37	98.9	0.54 ± 0.12	90.2

*The reaction mixture (2.0 ml) contained ATP, 2.25×10^{-3} M; $MgCl_2$, 3.5×10^{-3} M; tris buffer, 100 mM (pH 7.95); protein (the high-speed supernatant [105,000 × g for 60 min] of crude cell-free extract isolated from L1210 cells either in vitro or in vivo), 4.0 mg; and 2-thio-6-azauridine, different concentrations (0–50 μg). The reaction system was incubated at 37 C for 60 min with continual gentle shaking. The OMP decarboxylase reaction was started by the addition of 0.1 ml of OMP-carboxyl-^{14}C (0.2 mM, 3.5×10^4 cpm) to the reaction mixture and incubation at 37 C. At the appropriate time (10, 20, and 30 min), 0.5-ml amounts of the samples were pipetted into tubes containing 10% trichloroacetic acid (TCA) and were heated in a boiling water bath for 5 min to expel the last traces of $^{14}CO_2$. After cooling and centrifugation, 0.1 ml of the sample was used for the radioactivity determination.

†SA = specific activity (units of enzyme/mg of protein). A unit of OMP decarboxylase activity has been defined by Lieberman et al (10) as the amount of enzyme required to decarboxylate 1 mμM of OMP-7-^{14}C/hr calculated from the negative slope of initial reaction rate (10–30 min) and corrected via linear regression.

TABLE 3.—Cytotoxicity against L1210 cells in culture*

Drugs	Cytotoxicity (μg/ml)	
	ID50	ID90
2-Thio-6-azauridine	0.06–0.11	0.23–0.46
2-Thio-6-azauracil	26.0	48.0
6-Azauridine	0.05	0.11
6-Azauracil	> 50.0	—

*Five-ml aliquots of cells (about 5×10^3 cells/ml) were incubated in sterilized screw-cap culture tubes with and without drugs at 37 C for 3 days. Cell growth was determined on a model-A Coulter counter and % inhibition was calculated from comparison with controls (no drug).

dine (0.8 mM/kg) for 9 days yielded a 29% ILS. A direct comparison of the antitumor effects of 2-thio-6-azauridine and 6-azauridine was made. At equimolar doses, 2-thio-6-azauridine is somewhat less effective and less toxic (in terms of weight loss) than 6-azauridine (table 4). The lack of an observed dose response with 2-thio-6-azauridine may reflect its relative insolubility.

Effect of 2-thio-6-azauridine on macromolecular synthesis of L1210 leukemia.—As shown in table 1, no significant effect was observed on the incorporation of thymidine-^{3}H into DNA, uridine-^{3}H into RNA, or leucine-1-^{14}C into protein when the cells were incubated with 2-thio-6-azauridine (at up to 50 μg/ml for up to 4 hours). However, when the cells were incubated with 2-thio-6-azauridine at a level of 2 μg/ml, orotic acid incorporation was inhibited over 60%, and inhibition appeared to be dose-related. 2-Thio-6-azauracil showed little inhibitory effect. These results almost parallel those of cell-growth inhibition described in table 3. In leukemic BDF_1 mice, similar results were obtained when 2-thio-6-azauridine was administered ip at a dose of 200 mg/kg. Only orotic acid incorporation was significantly inhibited (from 70% to 90%) in all of the tissue studied (data not presented).

Effect of various metabolites on 2-thio-6-azauridine-inhibition of orotic acid incorpora-

TABLE 4.—Antitumor activity of 2-thio-6-azauridine and 6-azauridine against L1210 leukemia in vivo*

Dose† (mM/kg/day)	2-Thio-6-azauridine			6-Azauridine		
	Mean survival ±SD‡ (days)	%ILS§	Average weight change (g/mouse)¶	Mean survival ±SD (days)	%ILS	Average weight change (g/mouse)
0	8.0 ± 0.5	—	+1.2	8.0 ± 0.5	—	+1.2
0.8	10.3 ± 1.6	29	0	10.4 ± 1.0	30	−0.3
1.2	10.1 ± 0.7	26	−0.1	11.2 ± 1.9	40	−0.5
1.6	9.8 ± 0.8	23	−0.2	11.9 ± 0.9	49	−1.5

*Mice were inoculated ip with 1×10^5 ascitic L1210 cells/mouse on Day 0.
†Treatment (ip) was initiated on Day 1 and continued daily for 9 days.
‡SD = standard deviation.
§% ILS was calculated from mean survival rates of control and treated groups (eight mice per group).
¶Animals were weighed on Day 0 and again on Day 5.

TABLE 5.—Effect of various metabolites on the inhibition of orotic acid incorporation by 2-thio-6-azauridine in L1210 leukemia in vitro and in vivo

System	Organ	Metabolite	Specific activity (cpm/μg of RNA)		2-Thio-6-azauridine/controls (%)
			Control	2-Thio-6-azauridine	
L1210 cells in culture*		None	9820	3570	36.4
		Uridine	8220	7800	95.0
		Cytidine	8580	5320	62.0
		Deoxyuridine	9700	3200	33.0
		Deoxycytidine	9700	3220	33.2
L1210 leukemic mice†	Ascitic cells	None	1476	152	10.3
		Uridine	590	612	104.0
	Liver	None	459	117	25.5
		Uridine	464	216	46.6
	Spleen	None	275	84	30.5
		Uridine	167	201	120.0

*For the in vitro study, L1210 cells (about 5×10^5 cells/ml) were incubated at 37 C simultaneously with 2-thio-6-azauridine (1 μg/ml of cells), orotic acid-^{3}H (1.5 millicuries/18 mM/ml), and a given metabolite (5 μg/ml of cells) for up to 4 hrs. The rest of the procedure is described in table 1.

†For the in vivo study, L1210 leukemic mice (bearing about 1×10^6 cells/animal) were injected ip with 0.5 ml of samples composed of 4 mg of 2-thio-6-azauridine (or 0.9% NaCl), 20 mg of uridine (or 0.9% NaCl), and orotic acid-^{3}H (10 millicuries/2 mg). Animals (three mice per group) were sacrificed 1 hr later, and ascites, liver, and spleen were collected. The rest of the procedure is described in table 1. The doses of 2-thio-6-azauridine and uridine used in this study were 200 mg/kg and 1 g/kg, respectively.

tion into L1210 leukemia in vitro and in vivo, and of L1210 cell growth in culture.—The inhibition by 2-thio-6-azauridine of orotic acid incorporation into L1210 cells growing in culture is greatly reduced by the simultaneous addition of uridine (uridine:2-thio-6-azauridine = 5:1 wt/wt) and, to a lesser extent, by the addition of cytidine. Neither adenosine, guanosine (LH Li, unpublished data), deoxyuridine, nor deoxycytidine had any significant effect, as summarized in table 5. A similar phenomenon was observed in L1210 leukemic mice (table 5). When animals simultaneously received a dose of uridine (1 g/kg), the inhibition of orotic acid-^{3}H incorporation by 2-thio-6-azauridine (200 mg/kg) was completely prevented in ascitic cells and the spleen but was only partially reduced in the liver. Similar results, as seen in figure 1, were also obtained in the study of L1210 cell growth in culture. In the presence of uridine (5 μg/ml), the inhibition of cell growth by either 2-thio-6-azauridine or 6-azauridine (1 μg/ml) is almost completely eliminated.

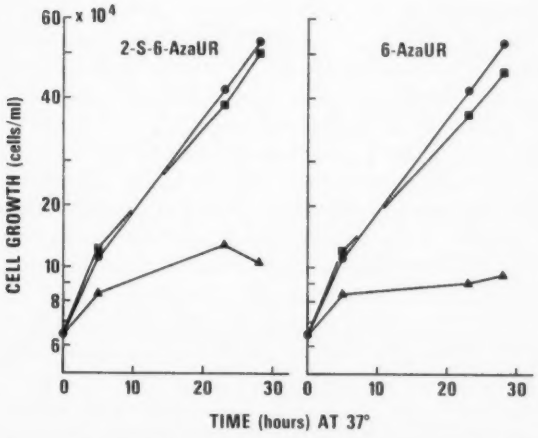

FIGURE 1.—Effect of uridine on the inhibition of L1210 cell growth in culture by 2-thio-6-azauridine and 6-azauridine. Four-ml aliquots of cells (about 6 × 10⁴ cells/ml) were pipetted into sterilized plastic culture tubes fitted with screw caps. The experiment was initiated by the simultaneous addition of 0.5 ml of medium or 2-thio-6-azauridine or 6-azauridine (10 μg/ml) and 0.5 ml of medium or uridine (50 μg/ml) and incubation at 37 C. Cell growth was determined (average of triplicate samples) by means of electronic particle counting. ●—● = Controls; ▲—▲ = 2-thio-6-azauridine or 6-azauridine (1 μg/ml); ■—■ = drug + uridine (5 μg/ml).

Effect of 2-thio-6-azauridine on the transformation of orotic acid in L1210 leukemia in vitro and in vivo.—Cells were exposed to the drug (5 μg/ml) for 1–4 hours (37 C) and acid-soluble fractions were isolated and separated by paper chromatography with a solvent system composed of 95% ethanol:1 M ammonium acetate (5:2), pH 7.5. The results in figure 2*A* indicate that although 2-thio-6-azauridine does not affect the entrance of orotic acid into the cells, it does significantly inhibit the transformation of orotic acid into phosphorylated products of uridine (apparently uridine-5′-triphosphate is the main product). In leukemic BDF_1 mice, as in the in vitro situation, the formation of phosphorylated uridine from orotic acid is also blocked by this drug, as shown in figure 2*B*.

Effect of 2-thio-6-azauridine on OMP decarboxylase activity and on the uridine kinase of L1210 cells in culture and/or in vivo.—The degree of phosphorylation of 6-azauridine-^{3}H by the crude cell-free extract isolated from L1210 cells growing in culture was determined by 30- and 60-minute periods of incubation at 37 C. Based on these data, a preincubation time of 60 minutes was chosen for determining the effect of 2-thio-6-azauridine at different concentrations on OMP decarboxylase under the assay conditions described in table 2. The reaction was started by the addition of OMP-carboxyl-^{14}C and was stopped after 30 minutes' incubation (within the initial rate of reaction). The results, as shown in table 2, indicate that 2-thio-6-azauridine, or its metabolic products or both, indeed inhibited the OMP decarboxylase activity of cell-free extracts which were isolated from L1210 cells growing either in culture or in leukemic BDF_1 mice. At the 25-μg/ml level, about 58% and 79% of enzyme activity was inhibited, respectively, and no apparent inhibition was observed without preincubation. Furthermore, no significant effect on the uridine kinase of L1210 cells in culture was observed in the presence of up to 200 μg/ml of either 2-thio-6-azauridine or 6-azauridine (LH Li, unpublished data).

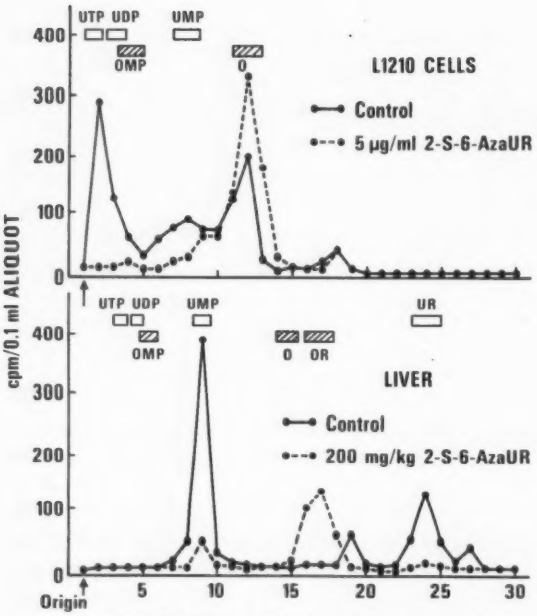

FIGURE 2.—Effect of 2-thio-6-azauridine on the transformation of orotic acid in L1210 leukemia in vitro and in vivo. For in vitro study, cells were exposed to the drug (5 μg/ml) and orotic acid-^{3}H (1.5 millicuries/18 mM/ml) for 1–4 hrs (37 C), and acid-soluble fractions obtained by extracting the cell pellet with 0.2N PCA (4 C) were separated by paper chromatography with a solvent system composed of 95% ethanol:1 M ammonium acetate (5:2), pH 7.5. For in vivo study, leukemic mice (bearing about 1 × 10^6 cells/animal) were injected ip with 0.5 ml of samples composed of 4 mg of 2-thio-6-azauridine (0.9% NaCl) and orotic acid-^{3}H (10 millicuries/2 μg). Animals (three mice per group) were sacrificed 1 hr later, and livers were collected. Acid-soluble fractions were obtained by extracting the tissue pellet with 0.2N PCA (4 C). To the acid extract containing the free nucleotides 1 mM of UMP was added as marker. The concentration of PCA was adjusted to 1N and the solution heated at 100 C for 20 min. Under these conditions all free uridine nucleotides should be quantitatively converted to the monophosphate (13). The hydrolysates were neutralized with KOH and PCA was removed as potassium perchlorate. Supernatants were lyophilized and redissolved in 0.5 ml of water just prior to paper chromatography with the solvent system 95% ethanol:1 M ammonium acetate (5:2), pH 7.5.

DISCUSSION

2-Thio-6-azauridine was found to be active against L1210 leukemia in vitro and in vivo. On a molar basis, it is about 100 times more active against L1210 cells growing in culture than its corresponding base, 2-thio-6-azauracil (table 3). In an attempt to understand the nature of its activity, the effect of 2-thio-6-azauridine on macromolecular synthesis was studied. For up to 4 hours' incubation, no significant effects on thymidine-^{3}H, uridine-^{3}H, and leucine-1-^{14}C incorporation into their respective macromolecules were observed, but orotic acid incorporation was significantly inhibited even at a level of 2 μg/ml (table 1). This phenomenon was also seen in vivo. These results collectively suggest that 2-thio-6-azauridine inhibits tumor cell growth by interfering with de novo synthesis of pyrimidine nucleotides. The inhibition of orotic acid incorporation into L1210 cells growing in culture (table 5) was almost completely prevented by the simultaneous addition of uridine (uridine:2-thio-6-azauridine = 5:1, wt/wt) and partially prevented by cytidine. Neither adenosine, guanosine, deoxyuridine, nor deoxycytidine had any effect. Similar results were obtained in studies of L1210 cell growth in culture (fig 1) and of orotic acid incorporation into the spleen and ascitic L1210 cells of leukemic BDF_1 mice (table 5). The inhibition of orotic acid incorporation into the liver, however, was only partially prevented by uridine, a phenomenon which remains to be explained. These results suggest that the phosphorylated product rather than the parent compound, 2-thio-6-azauridine, is responsible for its cytotoxic effect on tumor cells. Phosphorylation of 2-thio-6-azauridine probably occurs via the uridine kinase system (11) since only uridine and, to a lesser extent, cytidine prevented 2-thio-6-azauridine inhibition of both orotic acid incorporation (table 2) and cell growth (fig 1). The affinity of uridine (or cytidine) for uridine kinase may be much stronger than that of 2-thio-6-azauridine, hence no significant effect of this drug on the phosphorylation and incorporation of uridine (table 1) would be observed. Whether this drug is phosphorylated only to a monophosphate, as in the case of 6-azauridine (12), or is phosphorylated further to diphosphate and triphosphate derivatives and is ultimately incorporated into polynucleotides remains to be determined when a radioac-

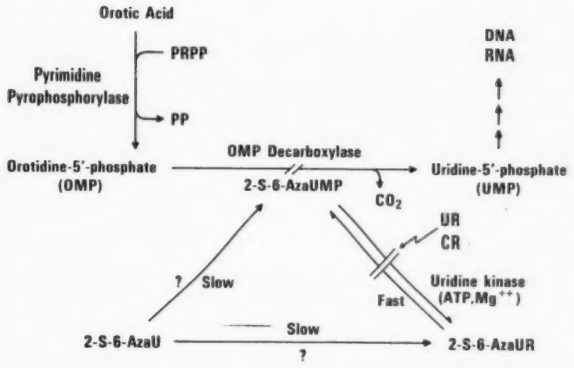

FIGURE 3.—A possible mechanism of action of 2-thio-6-azauridine on L1210 leukemia.

tive drug is available. Incorporation seems unlikely since the inhibition of L1210 cell growth in culture can readily be reversed by either the addition of uridine or by the removal of the drug after a period of 2 hours of contact with cells (LH Li, unpublished data).

2-Thio-6-azauridine was found to significantly inhibit the in vitro and in vivo transformation of orotic acid into phosphorylated products of uridine (fig 2). This again suggests that the active metabolite of this drug is its 5′-phosphate ester. Further evidence suggesting that 2-thio-6-azauridine-5′-phosphate is the actual inhibitor was obtained by studying the decarboxylation of orotidylic acid in an unfortified reaction mixture. By combining the reaction mixture for assaying nucleotide kinases (8), after a 60-minute preincubation period to allow the phosphorylation of 2-thio-6-azauridine to take place, with the OMP decarboxylase system, a marked inhibition of this enzyme is observed in cells obtained from culture and leukemic mice (table 2). 2-Thio-6-azauracil, the base, has considerably less cytotoxic activity (table 3). Apparently, conversion of 2-thio-6-azauracil to 2-thio-6-azauridine-5′-phosphate (via the pathways normally followed by uracil to its ribonucleotide) is a rather slow process. Thus, all evidence (summarized in fig 3) suggests that after conversion to the 5′-phosphate, 2-thio-6-azauridine, as does 6-azauridine (12,14), interrupts the de novo synthesis of pyrimidine nucleotides by specific inhibition of the enzyme OMP decarboxylase.

So far, our results indicate that the mechanism of action of 2-thio-6-azauridine against L1210 leukemia is more or less similar to that of 6-azauridine, a clinically useful drug in the treatment of psoriasis, mycosis fungoides, and certain types of tumors such as chronic myelogenous leukemias and acute leukemias (15,16). However, 2-thio-6-azauridine is somewhat less active against L1210 leukemia both in culture and in vivo than 6-azauridine (tables 3 and 4). Whether this drug possesses any other desirable characteristics remains to be determined.

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Estradiol Mustard (NSC-112259) and Phenester (NSC-116785): Possible Mediation of Action by Estrogen Binding Protein [1,2,3]

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SUMMARY

The potential of estrogen binding proteins in the cytoplasm to mediate the action of the steroidal alkylating agent, estradiol mustard, was investigated using competition studies of this drug for specific binding sites on the receptor. Estradiol mustard inhibited the specific binding of ^{3}H-estradiol-17β to uterine cytosol receptors while the alkylating moiety, phenester, was without effect. Inhibition of binding by estradiol mustard was observed at greater than 10^{-6} M and was complete at 10^{-3} M. From these data an I_{50} of 3×10^{-5} M was determined and a K_d of 3×10^{-6} M was calculated for estradiol mustard-receptor complex. Sucrose gradient analysis indicated that estradiol mustard blocked the formation of the specific 8S ^{3}H-estradiol-17β-receptor complex of the cytoplasm. The displacement of binding of ^{3}H-estradiol-17β by estradiol mustard observed both on sucrose gradients and in the dextran-coated charcoal assay is not due to liberation of estradiol resulting from breakdown of estradiol mustard.

[Cancer Chemother Rep 58:353–357, 1974]

[1] Received Jan 24, 1974; revised Apr 18, 1974; accepted Apr 18, 1974.

[2] Supported in part by institutional grant IN-18L from the American Cancer Society, and Public Health Service grant CA-11198 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Estradiol mustard: CAS reg. No. 22966-79-6; estra-1,3,5(10)-triene-3,17β-diol, bis[[*p*-[bis(2-chloroethyl)amino]phenyl]acetate] (lot No. 9306-CYP-50B, Upjohn Co.). Estradiol mustard was reported to contain no free estradiol.

Phenester: CAS reg. No. 31386-35-3; acetic acid, [*p*-[bis(2-chloroethyl)amino]phenyl]-, ethyl ester (lot No. 9306-CYP-32A, Upjohn Co.).

Both drugs were obtained courtesy of Dr. D. Jane Taylor, Breast Cancer Program Coordinating Branch, Division of Cancer Biology and Diagnosis, NCI.

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Estradiol mustard (fig 1), a steroidal alkylating agent comprised of an estradiol-17β nucleus with two cytotoxic chlorphenacyl side groups, was developed by Wall et al (1) from the concept of Larionov et al (2). Recently reported results from our laboratory indicate that estradiol mustard is moderately uterotrophic and causes regression of the R3230AC mammary adenocarcinoma of the rat (3). Vollmer and co-workers have suggested that the steroidal portion might act as a carrier of the cytotoxic moiety, facilitating its passage across cell membranes perhaps even to specific sites of hormonal action (4), by utilizing the hormone receptors in the target tissue (5). This hypothesis, although proposed, has not been examined experimentally. By analogy to estradiol-17β, mediation of the action of estradiol mustard by estrogen-binding protein would involve initially binding to the cytoplasmic estrogen receptor.

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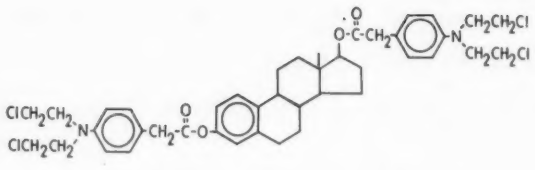

FIGURE 1.—Structure of estradiol mustard.

Accordingly, this study reports determinations of the ability of estradiol mustard to bind to receptors in rat uterine cytosol as measured by the inhibition of binding of the active estrogen, estradiol-17β.

MATERIALS AND METHODS

Estradiol-17β (estra-1,3,5[10]-triene-3, 17β-diol), tritiated either as ^{3}H-[2,4,6,7]-estradiol-17β or ^{3}H-[6,7]-estradiol-17β, with specific activity ranging from 40 to 114 curies/mM, was obtained from the New England Nuclear Corporation and used without further purification. Unlabeled estradiol-17β was obtained from Calbiochem. The nonsteroidal compound CN-55, 945-27 [1-(2-[*p*-(*α*-[*p*-methoxyphenyl]-*β*-nitrostyryl)phenoxy]ethyl)-pyrolidine, monocitrate] was obtained from Dr. J. R. Reel, courtesy of Parke-Davis Co.

Uteri were removed from 80–130-g Fischer 344 rats, cleaned of connective tissue, blotted, and homogenized in 10 mM tris HCl, 1.0 mM Na_2EDTA, 250 mM sucrose, pH 7.4 or 8.0 buffer, and a 50,000 or 105,000 × *g* supernatant was prepared as described by Wittliff et al (6). Each cytosol preparation was incubated for 10–30 minutes at 0–3 C with a test compound (unlabeled estradiol-17β, CN-55,945-27, estradiol mustard, or phenester) in the presence of buffer and 20% DMSO or 5% acetone which was used to dissolve the test steroid (total volume, 0.25 ml).[8] This mixture was then transferred to a shell vial containing the ^{3}H-estradiol-17β and incubated for a further 14–20 hours at 0–3 C. Alternatively, in some experiments all of the components were added simultaneously with similar results being obtained. To terminate the incubation and determine the amount of bound ^{3}H-estradiol-17β, a modified charcoal binding technique of Korenman (7), as described by Gardner and Wittliff (8), was used to remove the unbound ^{3}H-ligand, or gradient centrifugation analysis of bound ^{3}H-estradiol-17β was performed using a modified technique of Toft et al (9) as reported by Wittliff et al (6).

[8] Even in the presence of 20% DMSO or 5% acetone in the incubation mixtures estradiol mustard was insoluble at concentrations higher than 1×10^{-3} M.

RESULTS

For this study, we have confirmed the efficacy of the charcoal separation assay reported elsewhere (10) by demonstrating similar time, temperature, and ligand concentration relationships for ^{3}H-estradiol-17β binding to receptors from cytosol (results not presented).

Inhibition of ^{3}H-estradiol-17β binding is essentially complete at a hundredfold excess (2×10^{-7} M) of estradiol-17β (fig 2). Fifty percent inhibition ($[E_t]_{50}$) of binding was achieved by $5–6 \times 10^{-9}$ M unlabeled estradiol-17β. Estradiol

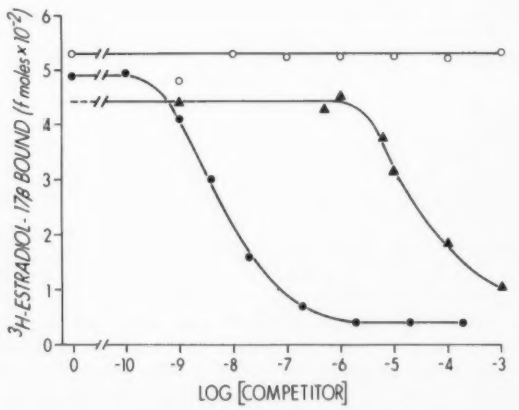

FIGURE 2.—Effect of estradiol mustard and its molecular components on the binding of ^{3}H-estradiol-17β to rat uterine cytosol receptor using the dextran-coated charcoal assay. Uterine cytosols were prepared and preincubated for 30 min at 0 C with the competitor dissolved in 5% acetone at the concentrations indicated in the figure. This mixture was then transferred to a shell vial containing ^{3}H-estradiol-17β (final concentration, 5.6×10^{-9} M) and incubated a further 16 hrs. The bound and free ^{3}H-estradiol-17β were separated as described in Materials and Methods. The protein concentration was 10 mg/ml. Each point is the mean of 3 independent determinations. Competitors: ●——● = unlabeled estradiol-17β, ▲——▲ = estradiol mustard, and ○——○ = phenester.

mustard, at concentrations greater than 10^{-6} M, was effective in inhibiting the binding of 5 nM ^{3}H-estradiol-17β. At 2–3 $\times$ 10^{-5} M estradiol mustard inhibition of ^{3}H-estradiol-17β was ~50% while inhibition of binding was essentially complete at 10^{-3} M (fig 2). Phenester, over a range of 10^{-10} to 10^{-3} M, did not affect the binding of ^{3}H-estradiol-17β to receptor. Using the following relationship devised by Sherman et al [9] it is possible to calculate a dissociation constant for the estradiol mustard-receptor complex.

$$K_c = \frac{K_E[C_t]_{50}}{[E_t]_{50}}$$

where K_c = the dissociation constant for the competitor (in this case, estradiol mustard); K_E = the dissociation constant for the ^{3}H-estradiol-17β, calculated to be 6 $\times$ 10^{-10} for rat uterine cytosol (data not presented); $[E_t]_{50}$ = the concentration of estradiol-17β that inhibits the binding of ^{3}H-estradiol-17β by 50%; and $[C_t]_{50}$ = the concentration of competitor that inhibits the binding of ^{3}H-estradiol-17β by 50%. Using this relationship, we have calculated a dissociation constant of ~3 $\times$ 10^{-6} M for estradiol mustard-receptor complex.

The sedimentation profile seen in figure 3 demonstrates the specificity of binding of ^{3}H-estradiol-17β to 8S cytosol receptors. These results confirm those previously published (11). An estrogen antagonist like CN-55, 945-27 completely inhibits the binding of ^{3}H-estradiol-17β to 8S receptors (fig 3*B*). As shown in figure 3*C*, 20% DMSO had no effect on the binding of ^{3}H-estradiol-17β to the receptor. Similar results were obtained when using DMSO with the charcoal adsorption assay and when binding was carried out with either assay in the presence of 5% acetone (results not presented). Phenester had no effect on the binding of ^{3}H-estradiol-17β (fig 3*D*) confirming the results presented in figure 2. Estradiol mustard at 1 $\times$ 10^{-5} M or 1 $\times$ 10^{-4} M reduced the binding of ^{3}H-estradiol-17β to 8S receptors by 35% and 80% respectively (fig 3 [*E* and *F*]). These reductions compare very well with those seen using the charcoal adsorption assay (compare figs 2 and 3[*E* and *F*]).

A time course of inhibition of ^{3}H-estradiol-17β binding by estradiol mustard was carried out at 4 C, in order to determine whether the inhibition observed was due to free estradiol

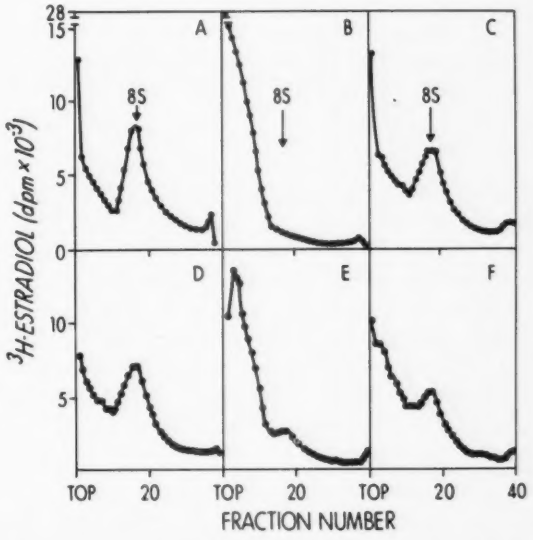

FIGURE 3.—Effect of estradiol mustard, its molecular components, and the antiestrogen CN-55,945-27 on the binding of ^{3}H-estradiol-17β to the 8S receptor of rat uterine cytosol separated by sucrose gradient analysis. Cytosol (0.15 ml) was preincubated for 10 min at 0–3 C with the competitor and then incubated a further 30 min in the presence of 3.0 $\times$ 10^{-9} M ^{3}H-estradiol-17β (total volume, 0.250 ml). The cytosols were then layered on linear 5%–20% sucrose gradients and centrifuged at 308,000 $\times$ g in a Beckman SW 56 Ti rotor for 8 hrs. Each tube was then pierced and 40 6-drop fractions were collected from the bottom of the gradient. The gradient profiles obtained for ^{3}H-estradiol-17β binding were as follows: ^{3}H-estradiol-17β in the presence of buffer alone (*A*) or 20% DMSO (*B–F*), plus 2 $\times$ 10^{-6} M CN-55,945-27 (*B*), buffer (*C*), 1 $\times$ 10^{-4} M phenester (*D*), 1 $\times$ 10^{-4} M estradiol mustard (*E*), and 1 $\times$ 10^{-5} M estradiol mustard (*F*). Using the computer program of Brooks and Wittliff (12), the area under the curve for the 8S peak was determined and expressed as percentage of the total radioactivity applied to the gradient: (*A*) 52%; (*B*) 12%; (*C*) 49%; (*D*) 52%; (*E*) 20%; and (*F*) 38%.

[9] Sherman MR, Atienza SBP, Shansky JR, et al. Progesterone receptors of chick oviduct. Steroid-binding "subunit" formed with divalent cations. Submitted to the *Journal of Biological Chemistry*, 1974.

TABLE 1.—Time course of the effect of estradiol mustard on the binding of ^{3}H-estradiol-17β to receptors in rat uterine cytosol*

Time (hrs)	^{3}H-Estradiol-17β specific binding		Inhibition of binding by estradiol mustard (%)
	Alone†	In presence of 1×10^{-5} M estradiol mustard†	
1	86305 ± 1946 (4)	67614 ± 1442 (4)	22
3	96392 ± 241 (4)	75611 ± 1286 (4)	22
5	98685 ± 1430 (3)	73833 ± 2772 (4)	25
13.6	97440 ± 411 (3)	74363 ± 3090 (4)	24

*Aliquots of rat uterine cytosol (0.2 ml) were incubated at 4 C with 4.5 nM ^{3}H-estradiol-17β (114 curies/mM) in the presence or absence of 1×10^{-5} M estradiol mustard and specifically bound ^{3}H-estradiol-17β was determined at the end of the incubation period using dextran-coated charcoal as described in Materials and Methods.

†Figures indicated are the mean ± SE with the number of samples indicated in parentheses.

resulting from the breakdown of estradiol mustard. The results are presented in table 1. The specific binding of ^{3}H-estradiol-17β to receptor sites in the uninhibited samples was maximal after 3 hours and remained unchanged for the duration of the incubation period. The inhibition of specific binding by 1×10^{-5} M estradiol mustard was not significantly different (range, 22%–25%) over the incubation period.

DISCUSSION

It is currently accepted that an essential characteristic of steroid hormone-responsive cells is the ability of the hormone to bind specifically to sites on proteins in the cytoplasm that are of high affinity but limited capacity. These "receptors" are then transferred by a sequential process to the nucleus where the ultimate effect of the hormone is expressed. Recent reports indicate that hormone antagonists block the action of natural hormones such as estrogens (11) or glucocorticoids (13). They can bring about these effects in one of two ways: (*a*) by competing with the natural hormone for uptake into the cell and thus preventing hormone entry, or (*b*) by preventing the binding of the hormone to its characteristic receptor. As steroid hormones enter their target cells, apparently by passive diffusion, the effect of antagonists would not be expected at the level of the plasma membrane. Accordingly, our studies with estradiol mustard, an estrogen analog (1,3), were carried out in vitro to determine the effect of this agent on the binding of the estradiol-17β to its cytosol receptor from rat uteri. The results of this study are in accord with those published by others using estrogen antagonists such as ethamoxytriphetol, clomiphene, CN-55,945-27, or nafoxidine (11). Estradiol mustard inhibits the binding of 5 nM estradiol-17β to its receptor at greater than 10^{-6} M. Estradiol mustard does not have as high an affinity for the estrogen receptor (ie, Ka $\sim$ 0.3 μM^{-1}) as some estrogen analogs or antagonists (7, 11). However, one selective advantage of estradiol mustard may be that the phenester moiety can interact chemically with the receptor maintaining the antagonist-receptor complex for a longer period of time. This maintenance of antagonist-receptor complex may further block the action of estradiol-17β by preventing the recycling of receptor as postulated by Munck et al (14).

It is possible that the inhibition of ^{3}H-estradiol-17β binding in vitro by estradiol mustard is the result of chemical or enzymatic hydrolysis of the estradiol mustard to free estradiol-17β and phenester. However, the results presented in table 1 do not support this conclusion since one would expect the cleavage of estradiol mustard to be time dependent. Studies currently underway with doubly labeled estradiol mustard indicate that it is the estradiol mustard and *not* free estradiol-17β liberated from the breakdown of the antagonist that is responsible for

the inhibition of binding.[10] Using the labeled estradiol mustard it will be possible to determine whether the mechanism of action of estradiol mustard on estrogen-target cells is similar to that reported by Turnell et al (13) for the antiglucocorticoid, cortexolone, on thymocytes.

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[10] Separation of uterine cytosol previously incubated with chromatographically purified $^{14}C/^{3}H$-labeled estradiol mustard ([6,7-^{3}H]-estra-1,3,5(10)-triene-3,17β-diol, bis[[p-[bis(2-chloroethyl-[1,2-^{14}C]-amino]phenyl]-acetate]) by centrifugation on linear sucrose gradients gave specific superimposable carbon-14 and tritium peaks.

Preliminary Report on the Treatment of Nonresectable Cancer of the Lung [1,2,3]

D. T. Kaung, [4] J. Wolf, [5] L. Hyde, [6] and M. Zelen [7]

Veterans' Administration Lung Cancer Study Group

SUMMARY

Between April 1969 and March 1972, 1546 patients who had nonresectable bronchogenic carcinoma received treatment according to the Veterans' Administration Lung Cancer Study Group protocols. Two hundred and thirty-nine of these patients had tumors confined to one hemithorax and were amenable to local supravoltage radiotherapy, chemotherapy, or both. Preliminary results indicated no definite overall superiority of one regimen over the others. Thirteen hundred and seven patients who had more extensive tumor involvement were treated with regimens consisting of high and low doses of L-asparaginase, NSC-45388, dibromodulcitol, hexamethylmelamine, procarbazine, CCNU, and streptozotocin. The results based on survival were compared with those of the concurrent standard reference regimen of intravenous cyclophosphamide. None of the newer regimens appeared to be superior to cyclophosphamide. Analysis of the background variables of patients indicated that initial performance status had a consistently strong influence on prognosis in the extensive disease group.

[Cancer Chemother Rep 58:359–364, 1974]

Since its inception in 1958, the Veterans' Administration Lung Cancer Study Group has studied a large number of chemotherapeutic agents, as well as radiotherapy, in the treatment of patients with nonresectable lung can-

[1] Received July 31, 1973; revised Nov 9, 1973; accepted Nov 19, 1973.

[2] This study was done under the auspices of the Research Service, Veterans' Administration Central Office, and the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), by the following participating investigators: *Joseph B. Mizgard*, Ann Arbor, Mich; *Richard W. Dwight* and *Robert M. Amick*, Boston, Mass; *Julius Wolf* and *Herbert E. Edlis*, Bronx, NY; *Sidney Sbar* and *Seymour Olshin*, Brooklyn, NY; *Richard M. Whittington* and *Levona W. Olmsted*, Cleveland, Ohio; *Hisashi Majima*, East Orange, NJ; *David T. Kaung*, Iowa City, Iowa; *Leroy Hyde*, Long Beach, Calif; *Albert Fink*, Los Angeles, Calif; *James Yee*, Martinez, Calif; *Frank S. Dietrich* and *John P. Griffin*, Memphis, Tenn; *Edward W. Humphrey*, Minneapolis, Minn; *Clyde O. Brindley*, Mountain Home, Tenn; *Stewart M. Scott* and *Frank K. Justice*, Oteen, NC; *Henry P. Close*, *Stewart McCracken*, and *Ralph M. Myerson*, Philadelphia, Pa; *John L. Fairly, Jr.*, Richmond, Va; and *Lyndon E. Lee, Jr.* (coordinator), Washington, DC.

[3] Drugs used in this study were supplied by DCT, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

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[5] Medical Service, VAH, Bronx, NY.

[6] Pulmonary Service, VAH, Long Beach, Calif.

[7] Statistical Laboratory, State University of New York at Buffalo, Amherst.

cer. Up to the present time, 13 protocols of controlled clinical trials have been used in sequence. This report outlines the preliminary results obtained from protocols 10, 11, and 12. Detailed accounts of various aspects of these studies are in preparation.

The general design of the study has been presented previously (1). All patients in the participating hospitals who had histologically or cytologically proven nonresectable bronchogenic carcinoma were admitted to the study. On admission, the patients were divided into limited disease groups and extensive disease groups according to the degree of their disease. In the limited disease group the tumor must be confined to one hemithorax. Local extension or nonpalpable positive ipsilateral supraclavicular nodes may be present if the entire tumor can be adequately treated by irradiation. All patients who did not meet the specific criteria for the limited disease group were treated in the extensive disease group. Previous studies from the Veterans' Administration Group had indicated the desirability of separating patients into the two groups during the evaluation of therapy (2–4).

In this study we used the percent of survival over time as the primary criterion for assaying effectiveness of treatment. Although randomization and large sample size tend to minimize intergroup differences, detailed analyses of background variables showed that the general clinical status and cell type were important factors affecting patients' responses to treatment.

TREATMENT REGIMENS

Protocol 10—initiated in April 1969

Limited disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Supravoltage radiotherapy, 4000–5000 rads in 4–6 weeks;

b. Cyclophosphamide, 0.3 g/m^2/ day intravenously (iv) × 5, followed by 200 mg/day orally;

c. Supravoltage radiotherapy, 4000–5000 rads in 4–6 weeks, plus cyclophosphamide, 200 mg/day orally.

Extensive disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Cyclophosphamide, 1.1 g/m^2 iv every 3 weeks;

b. NSC–45388, 325 mg/m^2/day iv × 5 (repeat course every 26 days);

c. High-dose L-asparaginase, 1500 IU/kg/wk iv × 6;

d. Low-dose L-asparaginase, 150 IU/kg/wk iv × 6;

e. Dibromodulcitol, 400 mg/m^2/wk orally × 6.

Protocol 11—initiated in February 1970

Limited disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Supravoltage radiotherapy, same as protocol 10;

b. BCNU, 185 mg/m^2 iv every 6 weeks.

Extensive disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Cyclophosphamide, same as protocol 10;

b. Dibromodulcitol, same as protocol 10;

c. Low-dose L-asparaginase, 150 IU/kg/wk iv × 6.

Protocol 12—initiated in June 1970

Limited disease group.—Same as protocol 11.

Extensive disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Cyclophosphamide, same as protocol 10;

b. L-Asparaginase, same as protocol 11;

c. CCNU, 130 mg/m^2 every 6–8 weeks;

d. Procarbazine, 150 mg/m^2/day orally;

e. Hexamethylmelamine, 8 mg/kg/day orally;

f. Streptozotocin, 1 g/m^2/wk iv × 10.

RESULTS

Limited disease group.—The median survival times of patients treated according to protocols 10 and 11 are shown in table 1. Cyclophosphamide or radiotherapy given alone appeared to be as effective as cyclophosphamide plus radiotherapy given in combination. BCNU also compared favorably with radiotherapy. When only squamous-cell carcinomas are con-

sidered, the radiotherapy group shows a significantly longer median survival time (60 ± 11.6 weeks) than the BCNU-treated group (25 ± 5.1 weeks). Since 52.5% of patients with well-differentiated squamous-cell carcinomas die with tumors still limited to the chest,[8] the data suggest that local irradiation is more effective than chemotherapy for this cell type. However, a similar group of patients who had squamous-cell carcinoma treated with the same radiotherapy regimen as in protocol 10 showed a median survival time of only 18.3 weeks, thus indicating the important influence that factors other than modalities of treatment have on survival. Similarly, although BCNU appeared to be more effective than radiotherapy for the cell type designated as "others," the histologic heterogeneity of the group and the relatively small number of patients studied preclude any meaningful interpretation.

Extensive disease group.—In this group of patients there was a consistent linear correla-

[8] Personal communication from Dr. Raymond Yesner, based on data from the Veterans' Administration Lung Group.

TABLE 1.—Median survival time (wks) by treatment and cell type: limited disease

		Protocol 10		Protocol 11	
Cell type	Treatment	Median survival time	Total No. of deaths/ total No. of patients	Median survival time	Total No. of deaths/ total No. of patients
All types	Cyclophosphamide	25.7 (5.7)*	20/23	27.4 (3.08)	79/89
	Radiotherapy	20.5 (4.5)	21/22		
	Radiotherapy + cyclophosphamide	21.8 (4.5)	23/25	27.9 (3.36)	69/80
	BCNU				
Squamous-cell carcinoma	Cyclophosphamide	10.0	8/9		
	Radiotherapy	18.3	10/10	60.1	27/32
	Radiotherapy + cyclophosphamide	33.7	8/9		
	BCNU			25.0	24/27
Small cell carcinoma	Cyclophosphamide	28.9	5/7		
	Radiotherapy	21.6	4/4	22.0	13/14
	Radiotherapy + cyclophosphamide	20.8	4/4		
	BCNU			27.9	18/20
Adeno-carcinoma	Cyclophosphamide	23.0	2/2		
	Radiotherapy	—	0/0	17.4	9/9
	Radiotherapy + cyclophosphamide	20.3	3/3		
	BCNU			22.0	9/10
Large cell carcinoma	Cyclophosphamide	29.8	1/1		
	Radiotherapy	13.6	3/3	32.7	11/13
	Radiotherapy + cyclophosphamide	—	0/0		
	BCNU			35.3	5/7
Others†	Cyclophosphamide	36.5	4/4		
	Radiotherapy	20.8	4/5	23.0	19/21
	Radiotherapy + cyclophosphamide	20.8	8/9		
	BCNU			59.2	13/16

*Standard deviation in parentheses.

†Disease confirmed by cytology only, or cases not easily classified.

TABLE 2.—Median survival time (wks) by treatment and cell type: extensive disease

Cell type	Treatment	Protocol 10		Protocol 11		Protocol 12	
		Median survival time	Total No. of deaths/ total No. of patients	Median survival time	Total No. of deaths/ total No. of patients	Median survival time	Total No. of deaths/ total No. of patients
All types	Cyclophosphamide	15.4 (1.6)*	96/101	11.7 (1.3)	78/81	13.1 (0.93)	200/212
	L-Asp (L)†	15.9 (2.6)	38/38	8.5 (0.9)	85/86	5.8 (0.9)	47/49
	L-Asp (H)†	4.3 (0.9)	23/24	—	—	—	—
	NSC-45388	10.0 (1.0)	95/100	—	—	—	—
	Dibromodulcitol	8.7 (1.0)	71/82	11.2 (1.8)	40/42	—	—
	CCNU	—	—	—	—	11.7 (1.3)	78/81
	Hexamethylmelamine	—	—	—	—	9.6 (1.1)	79/83
	Streptozotocin	—	—	—	—	9.6 (0.9)	115/124
	Procarbazine	—	—	—	—	10.0 (0.74)	182/194
Squamous-cell carcinoma	Cyclophosphamide	20.6	20/22	11.1	15/16	15.5	53/58
	L-Asp (L)	27.1	7/7	11.7	30/30	5.6	14/14
	L-Asp (H)	2.4	5/5	—	—	—	—
	NSC-45388	5.9	21/21	—	—	—	—
	Dibromodulcitol	7.8	16/18	6.1	8/8	—	—
	CCNU	—	—	—	—	11.6	22/22
	Hexamethylmelamine	—	—	—	—	8.1	25/25
	Streptozotocin	—	—	—	—	9.9	27/30
	Procarbazine	—	—	—	—	14.3	29/34
Small cell carcinoma	Cyclophosphamide	14.8	14/14	6.4	10/10	12.2	33/34
	L-Asp (L)	17.5	4/4	11.6	12/12	3.9	7/8
	L-Asp (H)	4.4	2/2	—	—	—	—
	NSC-45388	9.5	13/13	—	—	—	—
	Dibromodulcitol	4.5	7/8	3.5	5/5	—	—
	CCNU	—	—	—	—	9.2	13/13
	Hexamethylmelamine	—	—	—	—	7.7	17/17
	Streptozotocin	—	—	—	—	5.4	15/15
	Procarbazine	—	—	—	—	7.6	29/29
Adenocarcinoma	Cyclophosphamide	10.1	14/14	13.3	21/21	11.8	25/26
	L-Asp (L)	14.8	6/6	8.4	18/18	4.5	10/11
	L-Asp (H)	7.9	5/5	—	—	—	—
	NSC-45388	7.1	12/13	—	—	—	—
	Dibromodulcitol	5.5	12/12	13.1	8/8	—	—
	CCNU	—	—	—	—	10.8	12/14
	Hexamethylmelamine	—	—	—	—	11.0	5/6
	Streptozotocin	—	—	—	—	12.4	20/21
	Procarbazine	—	—	—	—	12.0	30/31

Large cell carcinoma	Cyclophosphamide	10.6	11/11	11.9	16/17	14.9	33/34
	L-Asp (L)	6.5	7/7	4.6	10/10	6.5	6/6
	L-Asp (H)	2.9	5/5	—	—	—	—
	NSC-45388	14.2	17/17	—	—	—	—
	Dibromodulcitol	11.3	10/11	6.4	5/5	—	—
	CCNU	—	—	—	—	10.2	12/13
	Hexamethylmelamine	—	—	—	—	10.5	15/16
	Streptozotocin	—	—	—	—	9.9	21/22
	Procarbazine	—	—	—	—	8.5	30/32
Others‡	Cyclophosphamide	18.7	37/40	12.5	16/17	12.6	56/60
	L-Asp (L)	8.4	14/14	7.2	15/16	7.7	10/10
	L-Asp (H)	3.9	6/7	—	—	—	—
	NSC-45388	16.5	32/36	—	—	—	—
	Dibromodulcitol	20.1	26/33	13.8	14/16	—	—
	CCNU	—	—	—	—	11.0	19/19
	Hexamethylmelamine	—	—	—	—	12.3	17/19
	Streptozotocin	—	—	—	—	11.6	32/36
	Procarbazine	—	—	—	—	9.3	64/68

*Standard deviation in parentheses.
†L-Asp (L) = low-dose L-asparaginase; L-Asp (H) = high-dose L-asparaginase.
‡Disease confirmed by cytology only, or cases not easily classified.

tion between log survival and the clinical condition of the patients (Karnofsky scale) (5) at the beginning of treatment, regardless of the therapeutic regimen used (6). The median survival times of patients treated with the various regimens are shown in table 2. The standard reference regimen of cyclophosphamide continued to do well when compared with the other treatment regimens. High-dose L-asparaginase was clearly an inferior regimen. Some regimens seemed to be more effective for specific cell types. The significance of the differences and the effects of important concomitant variables such as performance status, weight loss, and other factors on these differences will be discussed in detail in another report.

It is obvious from our studies that stratification according to cell types and extent of tumor involvement plus strict randomization did not insure uniformity of patient populations being compared. This undoubtedly was one of the major factors which caused the median survival time to fluctuate from year to year even when the same treatment regimen was used by the same hospitals. Conclusions based on studies of small numbers of patients (even though statistically significant) must be accepted with caution, since even the best available treatment is only slightly more effective than the others. Of the many patient variables which may affect the results of therapy, initial performance status exerted a consistent influence regardless of the type of therapy in the extensive disease group. This factor was therefore taken into account in protocol 13 in which patients were randomized separately into high- and low-performance status groups.

The overall results of therapy continue to be disappointing. Since combination and sequential therapies have been so successful in acute leukemias, lymphomas, and other solid tumors (7), treatment regimens employing these principles are being tried in protocol 13.

LIST OF COMPOUNDS

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Dibromodulcitol: NSC-104800; CAS reg. No. 10318-26-0; galactitol, 1,6-dibromo-1,6-dideoxy-; DBD

Hexamethylmelamine: NSC-13875; CAS reg. No. 645-05-6

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Streptozotocin: NSC-85998

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Response and Survival of Patients With Metastatic Neuroblastoma After Combination Chemotherapy With Adriamycin (NSC-123127), Cyclophosphamide (NSC-26271), and Vincristine (NSC-67574) [1,2]

Marco Gasparini, Franca Fossati Bellani, Renato Musumeci, and Gianni Bonadonna [3,4]

SUMMARY

Nineteen consecutively admitted patients who had stage IV neuroblastoma and were previously untreated were given combination chemotherapy with adriamycin, cyclophosphamide, and vincristine. The therapeutic effects and survival rates of these patients are reported. Although no patients achieved a complete remission, six patients achieved a partial response greater than 50% and five patients showed an objective improvement. Responders (11 of 19 or 59%) had a median survival time of 17.5 months and nonresponders had a median survival time of 5 months ($P<0.01$). The median survival time for both responders and nonresponders was 13.2 months. This figure is not statistically significant when compared with a similar group of 23 patients who were treated with single-agent chemotherapy given sequentially (7.5 months).

[Cancer Chemother Rep 58:365–370, 1974]

In the past few years several therapeutic approaches for the management of neuroblastoma have been reported (1–7). All reports advocate the usefulness of surgery (even if not radical), radiotherapy, and chemotherapy. In the apparently localized disease the combination of surgery plus radiotherapy is thought to be an adequate treatment (8–11). Available data suggest that chemotherapy neither increases survival nor decreases mortality (1,6,12,13). Although chemotherapy is the preferred treatment in the advanced and disseminated stages of neuroblastoma, some investigators do not agree on the real usefulness of chemotherapy in all forms of metastatic neuroblastoma (11,14). An adequate evaluation of retroperitoneal nodes, bone marrow, and liver in patients with neuroblastomas (especially those present in children older than 2 years of age) reveals that the majority have metastatic disease (13). Therefore, chemotherapy plays an important role in the different therapeutic approaches.

During the past few years cyclophosphamide and vincristine were found to produce objective responses of 28%–79% in patients when these agents were used singly through different dose schedules (2,4,15,16). The overall therapeutic responses produced by these agents used in combination ranged from 32% to 79% (1,3,6, 7,17). However, the regression obtained with these two drugs administered in sequence and in combination was not translated into an increased overall survival rate that was compa-

[1] Received Aug 2, 1973; revised Dec 4, 1973; accepted Dec 20, 1973.

[2] Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,-3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; CTX; EDX.

Vincristine: CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1); VCR.

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TABLE 1.—Site of metastases in 19 consecutive patients with neuroblastoma

Primary tumor site	No. of patients	Site of metastases						
		Bone	Bone marrow	Lymph nodes	Skin	Lungs	Liver	Ovaries
Thorax	2	1		1				
Abdomen	12	8	3	10	1	2	1	
Unknown	5	3		2	1			1
Totals	19	12	3	13	2	2	1	1

rable to that achieved in other neoplastic diseases of childhood (12,13).

During phase I and II studies with adriamycin using different dose schedules, prompt regressions were produced in 40%–80% of patients with diffuse neuroblastoma even if these patients were previously treated with conventional agents (18,19). For this reason, in 1971 our Institute began a prospective study which included the combination of adriamycin, cyclophosphamide, and vincristine in consecutive patients with metastatic neuroblastoma. The scope of this paper is to report the preliminary data concerning both responses and survival rates achieved with the above-mentioned combination.

PATIENTS AND METHODS

From January 1971 to December 1972, 19 consecutive patients (ten girls and nine boys) ranging in age from 7 months to 14 years were entered into the study. Seven patients were less than 2 years of age. All patients had diffuse biopsy-proven neuroblastoma that was untreated.

On admission, the extent of disease was documented through the following observations: chest roentgenogram, bone survey, intravenous (iv) urography, lower limb lymphography, liver scan, bone marrow aspiration, estimation of 24-hour urinary excretions of vanilylmandelic acid (VMA) and catecholamines. Abdominal arteriograms were taken for nine of 19 patients. After clinical and radiologic evaluations were conducted, all patients were classified as having stage IV disease. In the present series no patients were classified as stage IV-S (14). The site of metastases is reported in table 1.

Figure 1 outlines the treatment schedule employed in this trial. All drugs were administered by rapid iv injection. Radiation therapy with cobalt was employed only after three cycles of combination chemotherapy. The irradiation was delivered either to the site of initial bulky disease (usually the abdomen) or to one or more symptomatic sites for palliative purposes (eg, painful bone metastases). Tumor dosage ranged from 2000 to 3500 rads. Chemotherapy was usually started 2–4 weeks after diagnosis and was continued until clearcut

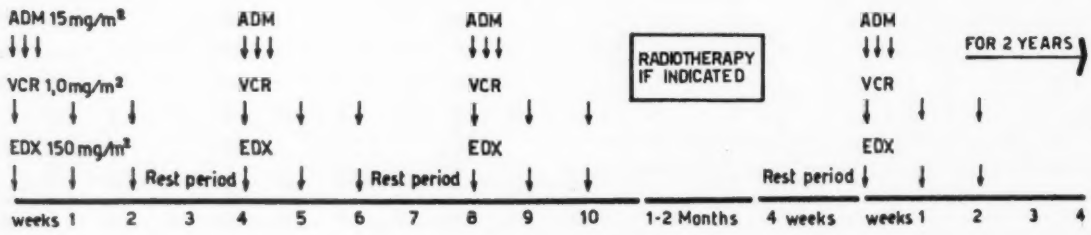

FIGURE 1.—Outline of the treatment schedule. ADM = adriamycin; VCR = vincristine; and EDX = cyclophosphamide.

evidence of disease progression occurred. At this point, either actinomycin D, BCNU, or CCNU was tried.[5]

The criteria for evaluating the degree of drug response were as follows: *complete response* = disappearance of all signs and symptoms of disease including recalcification of osseous metastases and a return to normal levels of VMA and catecholamines; *partial response* = 50% or greater decrease in the product of the longest perpendicular tumor diameters; and *objective improvement* = 25%–50% decrease in the longest perpendicular tumor diameters. Progression was defined as an increase of at least 25% in tumor measurements or the appearance of new lesions even in the presence of regression elsewhere.

Survival time from the beginning of therapy was calculated by the actuarial method. A similar analysis was conducted in 23 consecutive patients with diffuse neuroblastoma treated with single-agent chemotherapy given sequentially (cyclophosphamide, vincristine, actinomycin D, daunorubicin,[6] and adriamycin) from 1968 to 1970.

RESULTS

Table 2 reports the response to combination chemotherapy in relation to the site of primary and metastatic lesions. No patients achieved a complete remission in all parameters. Six patients showed a partial regression greater than 50%, and five patients showed a 25%–50% objective improvement. From this limited number of patients, only the abdominal, osseous, and nodal lesions can be evaluated. The primary abdominal tumor responded in 66% of the cases and six of 12 regressions were classified as greater than 50%. Response of the metastatic lymph nodes was observed in 38% of patients and in two patients a complete regression was obtained. In no patients was a recalcification of bone metastases observed. This is the main reason why no patients were classified as complete responders.

In responsive patients the initial regression occurred after the first cycle of treatment, and in a large majority of cases the maximum regression was achieved after the third cycle of treatment. The median duration of regression was 11 months (range: 2.5–20 months). At the time of this report, six of 11 responders are still alive while only one of eight nonresponders is alive. In responsive patients death occurred after a median time of 17 months (range: 9–20 months) from beginning of therapy, whereas death occurred in the unresponsive patients after a median time of 5 months (range: 2–13 months). Four of seven patients less than 2 years of age showed no response to treatment and their deaths occurred after a median time of 4 months.

VMA determination showed abnormal levels in a total of 12 of 19 patients (63%). In

[5] Actinomycin D: NSC-3053.

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

[6] Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6.

TABLE 2.—Response to chemotherapy in relation to site of lesions

Primary tumor site	No. of patients	No. of patients with response				Overall regression
		None	25%–50%	> 50%	CR*	
Thorax	2	1	1			50%
Abdomen	12	4	1	6	1	66%
Bone	12	12				0
Bone marrow	3	1			2	66%
Lymph nodes†	13	8	2	1	2	38%
Skin	2			1	1	0
Lung	2	2				0
Liver	1	1				0

*CR = complete remission.

†Peripheral and retroperitoneal.

unresponsive patients the levels of VMA remained essentially unchanged, whereas in all six responsive patients the abnormal levels of VMA decreased to normal.

In our group of patients treated sequentially with single-agent chemotherapy the median survival of responders was 9.2 months as compared with 4.3 months for nonresponders ($P < 0.01$).

Figure 2 reports the actuarial analysis of the survival time of responsive versus unresponsive patients. The median survival time of responsive patients (17.5 months) is more than three times longer than that of unresponsive patients (5 months). This difference is statistically significant ($P < 0.001$). By subtracting the time spent in remission, the survival time of responders becomes similar to that of nonresponders.

Figure 3 compares the overall actuarial survival time of all patients treated with combination chemotherapy (13.2 months) with the survival time of patients treated with single-agent chemotherapy given in sequence (7.5 months). There is a difference of 5 months in the median survival time between the two groups that are similar in terms of age and extent of disease. However, this difference is not statistically significant ($P < 0.5$).

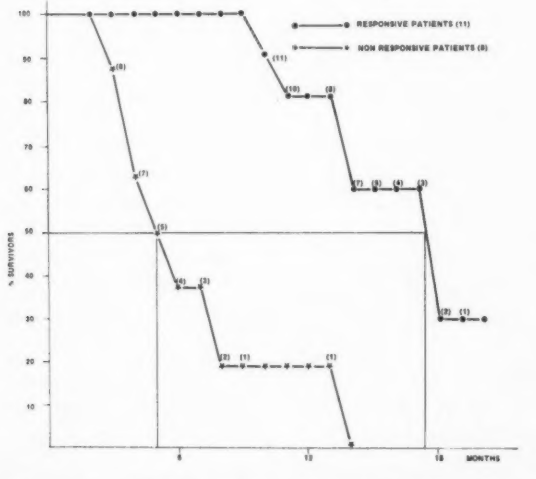

FIGURE 2.—Survival after combination chemotherapy with adriamycin, cyclophosphamide, and vincristine. In parentheses are the numbers of survivors.

Combination chemotherapy was usually well tolerated by the patients in this study. The most frequent toxic manifestation was alopecia which occurred in 15 of 19 (80%) patients. Leukopenia with a white blood cell (wbc) count that fell below 3000 cells/mm^3 occurred in all patients; however, only in one case was there a wbc count below 1000 cells/mm^3. Thrombocytopenia was always moderate, and in no instance did the platelet count fall below 50,000 platelets/mm^3. Bone marrow depression usually recovered during the interval between treatment cycles, so only occasionally was there a delay in continuing therapy. Nausea and vomiting, stomatitis, constipation, and paresthesia were always mild and never required a delay or a dose reduction between treatment cycles. There was no clinical, radiologic, or electrocardiographic evidence of cardiomyopathy. The mean total dose of adriamycin administered in this study was 260 mg/m^2 (range: 35–600 mg/m^2). Upon disease progression patients have failed to respond to subsequent treatment with either nitrosoureas or actinomycin D or both.

COMMENT

In the past decade there has been no substantial improvement in the management of neuroblastoma. Since the diagnosis is usually made after the neoplasia has become clinically obvious, neuroblastoma remains a difficult disease to cure (especially in children older than 2 years of age). Metastatic disease is also difficult to control beyond the sixth month from the beginning of therapy. With cyclophosphamide and vincristine given in combination, the median survival time from diagnosis was 6 months in the series reported by Sutow et al (12), 8 months in those series reported by Sawitsky et al (5), and 13 months in the series reported by Pinkel et al (1). Recently, Helson et al (7) have reported an overall response rate of 60% and a median survival time of 17 months from diagnosis by combining cyclophosphamide, vincristine, and daunorubicin administered after surgery plus radiotherapy. Although the dose of daunorubicin in Helson's series is equal in terms of mg/m^2 to the dose of adriamycin in

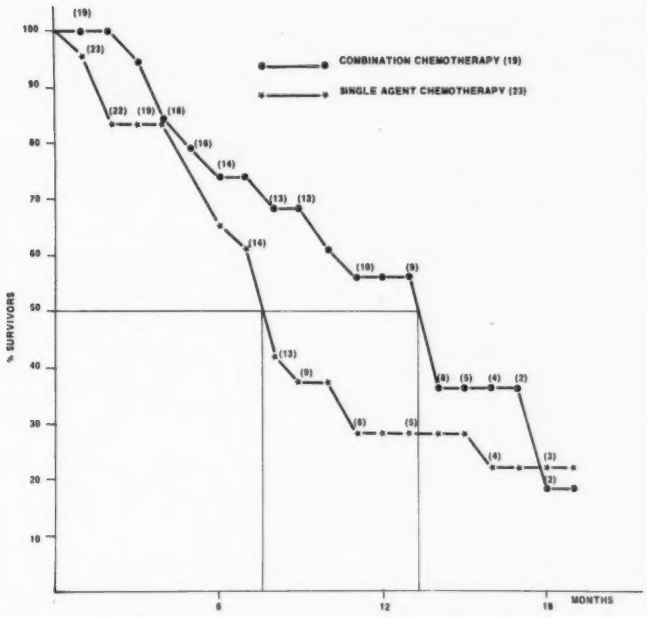

FIGURE 3.—Comparison of survival after combination chemotherapy (19 patients) and single-agent chemotherapy (23 patients). In parentheses are the numbers of survivors.

our series, the drug schedule as well as the doses of vincristine and especially of cyclophosphamide are different in the two therapeutic regimens. These successful and beneficial responses which could be partially attributed to the introduction of daunorubicin were long-lasting only in the patients under 2 years of age (nine of 20) at diagnosis.

In our series combination chemotherapy yielded a significantly increased survival rate in responders compared with that of nonresponders. This improved survival rate was not obtained through the achievement of complete remissions, since osseous recalcification was never observed in our group of patients. However, the fact that the subtraction of the time spent in remission from the group of responders yields a survival curve similar to that of nonresponders, definitely proves that combination chemotherapy was able to favorably affect the survival time. Radiation therapy which was not given to all patients did not contribute substantially to the increased survival time in responsive patients because it was delivered through limited fields and given mainly for palliative purposes. Although the number of children in this study is still too small to allow definitive conclusions, there is no particular difference within the group of responders in terms of sex and age distribution, site of primary tumor, and pattern of metastatic disease. The comparison of the survival curve of the whole group treated with combination chemotherapy to that of the group given single-agent chemotherapy fails to show in retrospect a statistically significant difference in the median survival time. Therefore, despite the limits of historical comparisons, we can not state on the basis of the available data that combination chemotherapy is superior to single-agent chemotherapy in terms of an increase in overall survival time and a decrease in percent of mortality. Although a more definitive answer for this particular point can be provided only through prospective controlled studies, our experience shows that combination chemo-

therapy which includes adriamycin has proved to be useful in controlling neuroblastomas in responsive patients.

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Effect of Hexamethylmelamine (NSC-13875) on Small Cell Carcinoma of the Lung (Phase II Study) [1,2]

Hiroshi Takita and Mukund S. Didolkar [3]

SUMMARY

The results of a phase II clinical trial of hexamethylmelamine in 23 patients with small cell carcinoma of the lung are presented. The overall response rate was 43%. The median survival time was 5.5 months and the mean survival time was 8.4 months. The most frequent side effects were nausea and vomiting; the hematopoietic toxicity was rather mild and transient. Because of relatively mild side effects, hexamethylmelamine should be further evaluated for treatment of small cell carcinoma in combination with other chemotherapeutic agents or with radiation therapy.

[Cancer Chemother Rep 58:371–374, 1974]

Hexamethylmelamine is one of the substituted melamines derived from cyanuric chloride. It was originally investigated for possible use in synthetic resin and synthetic fibers (1). Subsequently, an antitumor effect of hexamethylmelamine against Sarcoma 180 in mice was detected when ethylenimine derivatives and related compounds were screened in this tumor system (2). A phase I study conducted in 1965 showed effectiveness of hexamethylmelamine against carcinoma of the lung and ovary (3–5).

The chemical structure of hexamethylmelamine resembles that of the anticancer drug triethylenemelamine,[4] however, it has been suggested that hexamethylmelamine may not function as an alkylating agent but perhaps as a pyrimidine antimetabolite (6).

Our previous clinical study of this drug for various cell types of bronchogenic carcinoma revealed promising effectiveness against small cell carcinoma (7). The results of our extended phase II clinical study of hexamethylmelamine in 23 patients with small cell carcinoma of the lung are analyzed.

MATERIALS AND METHODS

The micronized form of hexamethylmelamine was supplied in capsules containing 100 mg of the drug. In most cases the total dosage was 12 mg/kg/day for 21 days administered orally in equal doses three times a day 1½–2 hours after meals. Prochlorperazine (Compazine) or chlorpromazine (Thorazine) was usually prescribed.

The following criteria were used in the selection of patients to be treated with hexamethylmelamine:

1. Histologically confirmed small cell carcinoma of the lung.
2. Ambulatory or semiambulatory at the start of the study.
3. White blood cell count (wbc) of 5000 cells/mm^3 or higher and platelet count of 100,000 cells/mm^3 or higher.
4. No radiation therapy or chemotherapy for at least 4 weeks.

All patients were periodically examined.

[1] Received Aug 21, 1973; revised Jan 7, 1974; accepted Jan 24, 1974.

[2] Hexamethylmelamine: CAS reg. No. 645-05-6. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute.

[3] Department of Thoracic Surgery, New York State Department of Health, Roswell Park Memorial Institute, Buffalo.

[4] Triethylenemelamine: NSC-9706; CAS reg. No. 51-18-3; s-triazine, 2,4,6-tris(1-aziridinyl)-.

Chest roentgenograms, liver scans, blood cell counts, and blood chemistry tests were performed at regular intervals.

The course of hexamethylmelamine was repeated at intervals of 6–12 weeks. Twenty-three patients were treated with one or more courses of the drug. In addition, four patients received a course of radiation therapy (3000 rads) to the primary lesion and the regional lymph node area, and one patient received radiation therapy for brain metastases.

RESULTS

Toxicity

Gastrointestinal symptoms in the form of nausea, vomiting, and anorexia were common and were observed in 15 patients (71%). Antiemetics usually controlled the symptoms, but in one case hospitalization was necessary to continue administration of hexamethylmelamine.

Leukopenia (3000–1000 cells/mm^3) was observed in eight patients. A gradual decrease in the wbc was noted 1 week after the start of the medication. The wbc reached its nadir at the third or fourth week after the start of therapy and was back to normal by the sixth week.

In two patients, the platelet count dropped below 100,000 cells/mm^3 (75,000 and 68,000 cells/mm^3) in the fourth week, but the counts returned to normal by the sixth week.

Up to nine courses of hexamethylmelamine were given but no cumulative toxic effects were observed. There were no neurologic symptoms or death due to the drug toxicity. No toxicity to the liver or kidney was observed.

Antitumor Effect (Table 1)

Of 23 patients, 21 had measurable lesions for evaluation of the response rate. Overall response rate (> 50% regression of the lesions) was 43% (nine patients). Three patients had a complete regression of the lesion and six patients had a greater than 50% regression of the lesion. Transient and less than 50% regression of the lesion was observed in two patients. There were ten nonresponders.

In this series the median survival time was 5.5 months (from the start of the therapy) and the mean survival time was 8.4 months.

Extent of Disease and Response Rate (Table 2)

There were five responders among ten patients with the disease localized in the thorax, and four of 11 patients with disseminated disease responded to hexamethylmelamine. In almost all cases, primary lesions as well as any metastatic lesions responded equally to hexamethylmelamine.

Previous Treatment and Response Rate

Three patients had previously received radiation therapy, two of whom responded. Among these three patients, there was one responder to hexamethylmelamine who also had previously responded to radiation therapy. Two patients had previously received cyclophosphamide [5] and both of them responded. One of these two patients responded to hexamethylmelamine.

Subsequent Treatment

Two of the nonresponders to hexamethylmelamine have subsequently received adriamycin (NSC-123127) and responded. They survived 13 and 14 months respectively (omitted from computation of overall survival).

DISCUSSION

Small cell carcinoma of the lung is known to carry a grave prognosis, and the overall 5-year

[5] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

TABLE 1.—Effect of hexamethylmelamine on small cell carcinoma (total No. of patients, 23)

No. of responding patients*				No. of unresponsive patients
100% regression	> 50% regression	< 50% regression	Regression not measurable	
3	6	2	2	10

*Response rate = 43%.

TABLE 2.—Effect of hexamethylmelamine on small cell carcinoma: extent of disease and response

Patient No.	Location of response in patients responding		
	100% regression	> 50% regression	< 50% regression
1	Primary, mediastinal nodes		
2	Primary, liver		
3	Primary, mediastinal nodes, and abdominal mass		
4		Primary, liver*	
5		Primary, mediastinal nodes	
6		Primary, liver	
7		Primary, mediastinal nodes	
8		Primary, mediastinal nodes	
9		Primary, supraclavicular nodes	
10			Primary, pleural effusion
11†			Skin

*Jaundice and ascites disappeared.

†Recurrence occurred after pneumonectomy. Mediastinal nodes and liver did not respond.

survival rate has been about 1% (8). At the same time, the disease is susceptible to various chemotherapeutic agents (9).

The overall median survival time of 161 patients treated at the Roswell Park Memorial Institute was 4.5 months (8), while there was an improvement in the median survival time of the patients who were treated with hexamethylmelamine (5.5 months from the start of therapy).

The response rate of small cell carcinoma in this series was 43%, which is in agreement with the report of compiled data of the National Cancer Institute; their data show a response rate of 36% among 67 patients with small cell carcinoma (10).

It is of interest that our previous study on the effect of CCNU [6] in 50 patients with carcinoma of the lung showed that none of the patients with disseminated disease (hematogenous metastasis) responded to the drug (11); on the contrary, four of 11 patients with hematogenous metastasis responded to hexamethylmelamine.

The most commonly observed side effects of the drug were nausea and vomiting (71%), while effects on the bone marrow were rather mild. According to the literature, hexamethylmelamine has been given in doses of 4–15 mg/kg/day for 21–90 days, but 12 mg/kg/day has been reported to be the maximally tolerated dose due to nausea and vomiting (3).

It is our opinion that one course given over 21 days is adequate in the treatment of small cell carcinoma since in all of our responders we observed regression of the lesion by the end of 21 days. It appears that the optimal interval of the course is 6–7 weeks since the length of

[6] CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

remission after a course of therapy in some of the responders was about 8 weeks.

As a single drug, hexamethylmelamine was found to be effective against small cell carcinoma of the lung, and because of relatively mild hematopoietic toxicity the drug should be further evaluated for treatment of small cell carcinoma in combination with other chemotherapeutic agents or with radiation therapy.

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Renal Dysfunction After Treatment With Isophosphamide (NSC-109724) [1,2,3]

Ralph A. DeFronzo,[4] Martin Abeloff,[5] Hayden Braine,[5] Richard L. Humphrey,[5] and Paul J. Davis [6,7,8]

SUMMARY

After treatment with isophosphamide (5 g/m^2), acute renal tubular acidosis, nephrogenic diabetes insipidus, phosphaturia, glucosuria, ketonuria, and generalized aminoaciduria developed in a patient with breast carcinoma. These findings represent drug-induced adult Fanconi syndrome. Nonselective proteinuria reaching the nephrotic range was also observed. The tubular and glomerular abnormalities normalized over several weeks, except for the persistence of a mild tubular acidification defect, vasopressin-fast concentrating defect, and mild proteinuria. Re-treatment of the patient with a lower dose of isophosphamide resulted in the reappearance of the major features of the syndrome. The effects of the two courses of therapy on tubular and glomerular function were not additive with a time interval between doses of 6 weeks.

[Cancer Chemother Rep 58:375–382, 1974]

Isophosphamide is a derivative of cyclophosphamide and has been shown to be effective against animal tumor systems resistant to cyclophosphamide and a variety of advanced malignancies in man (1). In high doses (50–80 mg/kg of body weight) this alkylating agent has resulted in azotemia, hematuria, and urinary frequency (1). In this report we describe a number of renal tubular defects, including aminoaciduria, glucosuria, renal tubular acidosis, and vasopressin-fast concentrating defect, that occurred after treatment with isophosphamide. Generalized tubular defects have not been reported after administration of isophosphamide or other alkylating agents.

CASE HISTORY

A 48-year-old woman with no family history of renal disease was admitted to the Oncology Division, Department of Medicine, Johns Hopkins University, for additional chemotherapy for advanced breast carcinoma. Twenty-six months before this admission, right radical mas-

[1] Received Sept 13, 1973; revised Nov 28, 1973; accepted Jan 8, 1974.

[2] Supported in part by Public Health Service grant CA-06973 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text. Isophosphamide was supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

[4] Clinical Physiology Branch, Gerontology Research Center, National Institute of Child Health and Human Development, Baltimore City Hospitals, Md.

[5] Oncology Division, Department of Medicine, Johns Hopkins University, Baltimore, Md.

[6] Endocrinology Division, Baltimore City Hospitals; and Johns Hopkins University, Baltimore, Md.

[7] The authors wish to thank Dr. Albert H. Owens, Jr., for his criticism of this manuscript.

[8] *Reprint requests to:* Dr. Richard L. Humphrey, Oncology Division, B2-S, Baltimore City Hospitals, Baltimore, Md 21224.

tectomy and bilateral oophorectomy had been performed. Fourteen months later, multiple tumor nodules appeared over the right anterior chest wall and upper arm and were treated with local radiotherapy. Tumor involvement of the left chest wall, left breast, and bilateral neck and supraclavicular nodes was apparent 16 months after diagnosis. The patient had a poor tumor response to combination chemotherapy [9] (8 weeks' duration) and to two courses of cytosine arabinoside (50 mg/kg of body weight, administered intravenously [iv] over 48 hrs). The latter treatment was given 12 weeks before the isophosphamide treatment. At that time renal function was normal: serum urea nitrogen (SUN) ranged from 6 to 15 mg/100 ml, serum creatinine was 0.6–1.0 mg/100 ml, and routine urinalysis (six times) revealed no cellular elements. Urine pH levels ranged from 5 to 6 (Labstix, Ames) and urine specific gravities were as high as 1.019–1.023. Qualitative testing revealed no glucosuria, ketonuria, or proteinuria. Serum chloride was 103–105 mEq/liter, bicarbonate was 25–27 mEq/liter, calcium was 9.0 mg/100 ml, phosphorus was 3.8 mg/100 ml, uric acid was 5.4 mg/100 ml, and serum albumin was 3.6 g/100 ml. Fasting serum glucose was 96–110 mg/100 ml.

Physical examination at the time of admission to our clinic showed a moderately obese, middle-aged, black woman with massive edema of both upper arms. Blood pressure was 150/80 mm Hg, heart rate was 80/minute, respirations were 15/minute, and oral temperature was 98.6 F. Multiple tumor nodules were present over the anterior chest wall with readily apparent bilateral, matted axillary, supraclavicular, and cervical node enlargement. The right breast was absent. There were ulcerating tumor nodules in the left breast. The remaining physical examination results were normal.

Initial laboratory data revealed normal renal function except for a 24-hour urinary protein excretion of 356 mg/day. SUN was 7 mg/100 ml, serum creatinine was 1.0 mg/100 ml, and creatinine clearance was 113 ml/minute. There were no abnormalities of urine sediment; glucosuria and ketonuria were absent and urine pH ranged from 5.10 to 6.12 (samples collected under mineral oil, pH measured with a Beckman Expandomatic pH meter). Twenty-four-hour urinary excretion of potassium, phosphorus, and calcium were within normal limits (fig 1). Urine uric acid excretion was elevated (1010 mg/24 hrs).

METHODS

Clinical data.—Complete 24-hour urine collections, except as noted, were made throughout the hospitalization. In the first course isophosphamide was administered iv over 1 hour at a dose of 5 g/m² (total dose, 9.5 g); a second course of 6.5 g (total dose) was carried out 34 days after the initial treatment. During the initial treatment period, allopurinol (300 mg/day) was administered with isophosphamide. Allopurinol is not known to have a primary

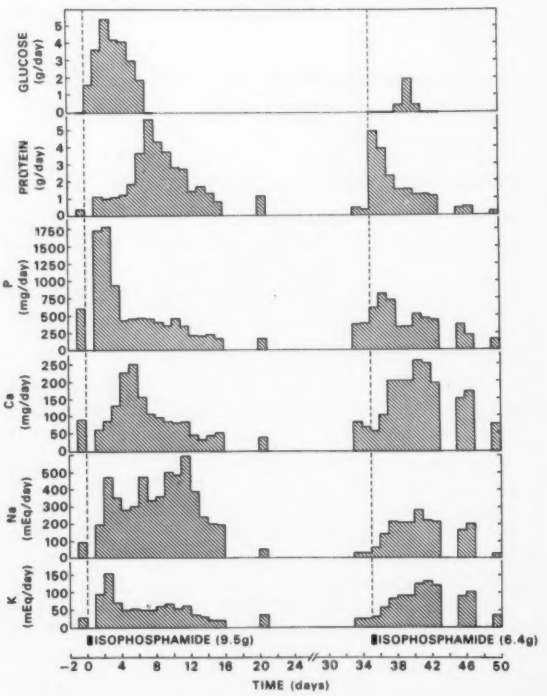

FIGURE 1.—Time sequence of changes during urinary excretion of potassium, sodium, calcium, phosphorus, protein, and glucose after treatment with isophosphamide.

[9] Prednisone, 30 mg/day orally; cyclophosphamide, 50 mg/day orally; 5-fluorouracil, 500 mg/week iv; methotrexate, 25 mg/week iv; and vincristine, 1 mg/week iv.

effect on renal function (2). In the second treatment course, the patient received no drugs in addition to isophosphamide.

Urine and serum electrolytes; urine glucose, ketones, and amino acids.—Sodium, potassium, chloride, bicarbonate, calcium, and phosphorus levels were measured on a Technicon AutoAnalyzer. Urine glucose was measured qualitatively (glucose oxidase, Labstix, Ames) and quantitatively (ferric chloride reagent). Urine and serum ketones were determined with Ketostix (Ames). Qualitative urinary amino acid analyses were performed on samples of selected 24-hour collections (Gilson high-voltage electrophoresis). Quantitative determinations of certain amino acids were also made (3).

Serum and urine protein determinations.—Serum total protein was measured by the method of Gornall et al (4), and urine total protein was measured by the method of Savory et al (5) with bovine albumin as the standard (6). Cellulose acetate membrane electrophoresis of serum and urine was carried out by the method of Laurell et al (7). Strips were stained with ponceau S in 5% trichloroacetic acid and cleared. Densitometry was performed using a Densicord 542 (Photovolt Corp., New York, NY). Immunoelectrophoresis was done in 1.5% Ionagar No. 2 (Colab Laboratories, Inc., Chicago Heights, Ill) using a 1:2 dilution of 0.04 M sodium barbital-0.07 M sodium acetate buffer (pH 8.2) for the agar preparation and undiluted buffer in the electrode chambers. The method was otherwise as described by Campbell et al (8). Specific antisera were obtained from Kallestad Labs, Inc., Chaska, Minnesota. Immunoglobulin quantitation was performed by the Mancini technique (9) using plates prepared by Kallestad Labs for quantitating human IgG, IgA, and IgM at normal, low, and ultralow levels (Quanti-plate™).

Acidification tests.—After collection of two baseline urine samples, 0.1 g of ammonium chloride/kg of body weight was administered to the patient either iv or orally. Urine and serum samples were obtained thereafter at 2-hour intervals for 14 hours. Arterial blood pH was determined prior to administration of ammonium chloride and at the nadir of serum bicarbonate. All urine samples were collected under mineral oil.

Urine concentrating ability.—Urine osmolality (U_{osm}) was measured cryoscopically after overnight dehydration of the patient (16 hrs), after intramuscular (im) administration of 5 units of Pitressin tannate in oil, and after iv administration of aqueous Pitressin (300 milliunits/hr for 5 hrs). Vasopressin from the same lot used in the patient had activity verified in control patients.

RESULTS

Clinical observations.—Immediately after the initial course of isophosphamide, the patient became disoriented, lethargic, and incontinent of stool and urine for 24 hours. Blood pressure and heart rate were unchanged. Lumbar puncture revealed an opening pressure of 260 cm H_2O but was otherwise normal. Except for Day 1, when urinary incontinence supervened, urine collections were complete. Hemorrhagic cystitis occurred on Day 2 and bloody urine persisted grossly until Day 9. Microscopic hematuria continued through Day 12.

Glomerular dysfunction.—Creatinine clearance decreased to 86 ml/minute by Day 2, reached a low of 64 ml/minute on Day 4, and returned to the pretreatment level by Day 11. Serum creatinine and SUN levels remained within normal limits. Proteinuria was maximal on Day 7 (5.60 g/24 hrs). Prior to isophosphamide treatment, cellulose acetate electrophoresis and immunoelectrophoresis of the patient's urine protein (concentrated × 75) were normal. On Day 1, however, there was significant proteinuria. The electrophoretic pattern from Day 6 demonstrated the presence of all electrophoretic fractions with albumin predominating. Immunoelectrophoresis revealed a marked increase in all immunoglobulin fractions including IgM. On Day 11 the electrophoretic pattern was similar, but IgM was no longer detectable at the 75× concentration. By Day 35 (immediately before the second course of isophosphamide) the 24-hour urine protein excretion had returned to the baseline level, but the electrophoretic pattern, immunoelectrophoresis, and immunoglobulin quantitations revealed a con-

tinued low level leak of IgG and IgA. This pattern of proteinuria recurred during the second course of isophosphamide. It should be noted that hemorrhagic cystitis occurred only during the first course of therapy (a fact which complicates interpretation of urine protein excretion) but was absent during the second course when significant proteinuria was qualitatively and quantitatively similar to that of the initial course.

Tubular dysfunction.—Renal tubular cells and granular casts were observed in the urine on the day after isophosphamide treatment.

Ketonuria, glucosuria, phosphaturia, potassium loss, aminoaciduria.—Ketonuria was noted on Day 2 with negative tests for serum ketones. Glucosuria appeared on Day 2 and persisted for 5 days (fig 1), during which time serum glucose ranged from 90 to 110 mg/100 ml (simultaneous urine and serum glucose measurements). Within 24 hours after isophosphamide treatment, urine excretion of phosphorus and potassium increased markedly (fig 1) and levels remained elevated for 4 days. Serum phosphorus decreased to 1.9 mg/100 ml and serum potassium decreased to 3.5 mEq/liter. Urine calcium excretion increased progressively but still remained within the normal range. Changes in serum and urine uric acid levels in the initial treatment course are not interpretable because of the administration of allopurinol prior to isophosphamide and the patient's elevated baseline uric acid excretion. Moderate increases in urinary amino acid excretion were noted on the first day after isophosphamide treatment. On Day 2 there was marked generalized aminoaciduria which persisted through the fourth day after treatment. By Day 2 there was a tenfold or greater increase in urine excretion of lysine-ornithine, alanine, valine, and glutamic acid-citrulline, and twofold to fourfold increases in urinary arginine, leucine-isoleucine, tyrosine, phenylalanine, aspartic acid, cystine, and methionine (table 1).

Acidosis and urine acidification defect.—By Day 2 after treatment serum bicarbonate had decreased to 12 mEq/liter and serum chloride had increased to 113 mEq/liter. Serum bicar-

TABLE 1.—Urine amino acid excretion (μmols/day) after treatment with isophosphamide

	Day								
Amino acid	0*	1	2	4	35†	36	38	42	49
Lysine-ornithine	74	240	1322	851	165	268	1151	1512	231
Arginine	90	208	293	203	94	119	164	135	58
Alanine	195	868	4531	1873	400	896	3124	3159	1983
Valine	74	201	925	428	94	268	164	594	77
Leucine-isoleucine	59	136	255	252	71	119	212	243	58
Tyrosine	74	140	250	330	71	149	186	189	116
Phenylalanine	82	126	184	245	71	119	212	216	77
Aspartic acid	72	190	245	103	118	149	45	1782	847
Glutamic acid-citrulline	205	1018	25,960	315	141	5802	30,732	29,692	12,589
Cystine	90	179	236	521	71	88	141	486	154
Methionine	59	108	123	86	47	59	45	54	39

*Isophosphamide, 9.5 g.
†Isophosphamide, 6.4 g.

TABLE 2.—Urinary acidification after treatment with isophosphamide

Day	Dose of ammonium chloride	Urine pH	Serum bicarbonate (mEq/liter)	Blood pH
0*	—	5.10	26	—
2	—	6.50	12	7.39
4	—	6.60	18	7.42
6	7.0 g iv	Before: 6.91	19	7.42
		After: 6.54	14	7.39
16	7.0 g iv	Before: 6.73	23	7.51
		After: 6.54	18	7.49
27	7.2 g orally	Before: 6.11	23	7.43
		After: 5.10	14	7.37
32	—	5.92	24	—
35†	—	6.11	25	—
39	—	6.43	16	—
41	7.0 g orally	Before: 6.38	18	7.45
		After: 5.20	14	7.41
68	6.5 g iv	Before: 6.95	22	7.47
		After: 6.21	17	7.42

*Isophosphamide, 9.5 g.
†Isophosphamide, 6.4 g.

bonate did not normalize until 13 days after isophosphamide. The appearance of acidosis may have blunted the development of hypokalemia despite the hyperkaluria described above. Inappropriately alkaline urine was noted during the development of hyperchloremic acidosis (table 2). On Days 6 and 16 after isophosphamide treatment, acidification tests were performed. Despite decreases in blood pH (7.39) and serum bicarbonate (14 mEq/liter), urine acidification was significantly impaired (minimum urine pH, 6.54). On Day 27 urine acidification following administration of ammonium chloride was demonstrated, but not until serum bicarbonate decreased to 14 mEq/liter suggesting the presence of a proximal tubule acidification defect.

Urine concentrating defect.—During the first 8 days after treatment the patient lost 10.9 kg of body weight, of which 6.5 kg were explained by urinary loss. Despite frank polyuria and volume depletion, U_{osm} failed to increase above 400 mOsm/kg. On the fourth day after treatment, 5 units of Pitressin given im increased U_{osm} to only 459 mOsm/kg (table 3). Thirty-four days after isophosphamide treatment the patient continued to demonstrate impaired urinary concentrating ability in response to dehydration and exogenous vasopressin.

Re-treatment with isophosphamide.—Because of significant tumor response to isophosphamide, the patient was re-treated with a lower dose (total dose, 6.4 g) 5 weeks after the initial course. Immediately before re-treatment, the patient demonstrated normal serum electrolytes, calcium, and phosphorus levels, and had a normal urinalysis. There was no aminoaciduria, glucosuria, or ketonuria, and urinary excretion of potassium, phosphorus, uric acid, and calcium were normal. Proteinuria had decreased to 330 mg/day with a small leak of albumin, IgG, and IgA (table 4). Despite the lower retreatment dose, isophosphamide administration again resulted in glucosuria, phosphaturia, generalized aminoaciduria, kaluria, and increased

TABLE 3.—Urine concentrating ability after treatment with isophosphamide

Day	U_{osm}: overnight dehydration	U_{osm}: after im dose of Pitressin tannate
0*		
4		439
5		459‡
12	453	—
16	355	454
25	512	—
26	—	616
34	609	—
35†	—	—
41	370	398
50	389	552
70	482	602
71	—	580

*Isophosphamide, 9.5 g.
†Isophosphamide, 6.4 g.
‡After aqueous Pitressin given iv at 300 milliunits/hr for 5 hrs.

proteinuria. The abnormalities persisted for 7–9 days. During the re-treatment course, hyperuricosuria was not obscured by allopurinol administration. Creatinine clearance fell from 113 to 62 ml/minute on the day after isophosphamide, remained at this level until the fifth day after treatment, and gradually returned to normal on Day 10. During re-treatment, the patient again showed a frank defect in urinary acidification. Serum bicarbonate fell from 25 to 16 mEq/liter and serum chloride increased from 101 to 111 mEq/liter. Urinary pH was measured daily and failed to decline below 6.38. When serum bicarbonate decreased from 18 to 14 mEq/liter with administration of ammonium chloride on Day 6 of the re-treatment course, urine pH decreased to 5.20. Although serum electrolyte levels normalized by Day 9, impaired urine acidification was still present 32 days after isophosphamide re-treatment, as was impaired response to vasopressin (tables 2 and 3). No tumor response was observed after this second course of isophosphamide.

DISCUSSION

The renal tubular defects we have observed after administration of isophosphamide consisted of generalized aminoaciduria, phosphaturia, glucosuria, urine potassium loss, and defective urinary acidification and concentrating functions. The defect in urine concentrating ability may be secondary to (*a*) defective sodium reabsorption in the loop of Henle with resultant solute diuresis and dissipation of the medullary osmotic gradient, (*b*) the inability of the collecting duct to respond to vasopressin, or (*c*) a combination of these. The hyperchloremic acidosis observed immediately following isophosphamide could represent proximal or distal renal tubular acidosis or both. However, the ability to normally acidify the urine on Day 27 (pH, 5.10) after a drop in the serum bicarbonate level to 14 mEq/liter suggests a proximal acidification defect. The inability to acidify the urine on Day 2 despite a lower serum bicarbonate level (12 mEq/liter) could represent a superimposed distal acidification defect or a more severe proximal defect that had partially corrected itself by Day 27. The ketonuria observed in our patient probably reflects a defect in organic acid reabsorption sometimes seen with the adult Fanconi syndrome (10); however, the development of systemic ketosis with decreased caloric intake immediately after isophosphamide treatment cannot be excluded.

TABLE 4.—Twenty-four-hour urinary protein excretion (mg) after treatment with isophosphamide

Protein	Day 0*	6	8	11	35†	36	55
Total protein	360	3640	4300	2740	330	4940	300
Albumin	150	1090	1550	1120	140	2320	130
IgG	3	830	650	320	50	430	20
IgA	< 2	140	100	60	3	90	2
IgM	0	19	8	6	0	8	0

*Isophosphamide, 9.5 g.
†Isophosphamide, 6.4 g.

The adult Fanconi syndrome has been observed after the use of two other antineoplastic agents (streptozotocin [11], a nitrosourea, and isophthalanilide [12], a terephthalanilide derivative) but not after isophosphamide or other alkylating agents. We have prospectively studied 22 patients receiving high-dose cyclophosphamide therapy ($\geq$ 50 mg/kg of body weight) and have not observed proteinuria, evidence of urine acidification defect, or alteration in tubular handling of glucose, potassium, phosphorus, or uric acid (13). The latter observations suggest that the development of the adult Fanconi syndrome in the patient reported here is a specific effect of isophosphamide. Furthermore, no renal dysfunction was observed in our patient after previous treatment with cyclophosphamide, methotrexate, vincristine, cytosine arabinoside, and 5-fluorouracil. We have since studied two additional patients receiving isophosphamide at a reduced dosage (3.5 g/m^2). Both manifested nonselective proteinuria similar to the patient described herein, but tubular dysfunction was not noted at this lower dosage. The tubular and glomerular defects which followed both courses of isophosphamide were largely reversible by 5 weeks; there appeared to be no cumulative effects of the two courses of therapy, although our patient was left with slight impairment of urinary acidification and concentrating ability.

The acute development of generalized tubular dysfunction after the use of isophosphamide resulted in significant management problems including acidosis (serum bicarbonate as low as 12 mEq/liter) and nephrogenic diabetes insipidus (with a resultant 6.5-kg weight loss due to inadequate fluid replacement). Glucosuria and ketonuria, occurring concomitantly in such patients, may lead to the mistaken diagnosis of diabetes mellitus.

LIST OF COMPOUNDS

Allopurinol: NSC-108836; CAS reg. No. 17795-21-0; 1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol, monosodium salt

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide

Isophthalanilide: NSC-53212; CAS reg. No. 5262-40-8; isophthalanilide, 4', 4"-di-2-imidazolin-2-yl-, dihydrochloride

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Streptozotocin: NSC-85998

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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BCNU (NSC-409962) in the Treatment of Advanced Hodgkin's Disease, Lymphosarcoma, and Reticulum Cell Sarcoma[1,2,3]

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SUMMARY

BCNU was administered to 44 randomized patients with advanced Hodgkin's disease, lymphosarcoma, or reticulum cell sarcoma; two schedules and two dose levels for each schedule were used. Nineteen of the 44 patients (43%) showed a therapeutic response; five patients had complete regressions and 14 had regressions greater than 50%. The breakdown of responses was as follows: 16 of 33 patients (48%) with Hodgkin's disease; two of seven with lymphosarcoma; and one of four with reticulum cell sarcoma. The responses did not vary significantly by dose or schedule. The duration of response varied from 40 to 771+ days. No evidence of cross resistance with prior alkylating agents, mechlorethamine and cyclophosphamide, was noted. The toxic reactions observed most often were bone marrow depression, nausea, and vomiting. There were two deaths related to toxicity. For the various regimens the median nadirs of leukopenia ranged from 1.9 to 3.1 $\times$ 10^3 cells/mm^3, and they occurred on medians of 30–43 days after the start of treatment. The median numbers of days to recovery from the nadirs were between 11 and 16 days. The equivalent figures for platelet toxicity and recovery were 28–51 $\times$ 10^3 cells/mm^3 (median nadirs), 24–42 days (median days of nadir occurrence), and 7–22 days (median days to recovery). Results indicate a definite role for BCNU, especially in the treatment of Hodgkin's disease.

[Cancer Chemother Rep 58:383–392, 1974]

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[1] Received Sept 17, 1973; revised Dec 17, 1973; accepted Jan 8, 1974.

[2] Presented in part at the meeting of the American Society of Clinical Oncology, San Francisco, Calif, Mar 22, 1969.

[3] BCNU: CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-. Supplied by Drug Research and Development (formerly Cancer Chemotherapy National Service Center), Division of Cancer Treatment, National Cancer Institute (NCI).

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[6] This study was done by the Acute Leukemia Group B (*James F. Holland*, chairman; *Oliver Glidewell*, statistician [Operations Office, CA-10456]) and the Eastern Cooperative Oncology Group (*Bruce I. Shnider*, chairman [*Paul P. Carbone*, current chairman]). The following institutions participated in the study: Acute Leukemia Group B—McGill University Hospitals (Royal Victoria Hospital), Montreal, Canada (*Louis Lowenstein* [deceased]); Medical College of Virginia, Richmond (*John H. Moon* [deceased], CA-03735); New York Hospital, Cornell Medical Center & Affiliates, New York (*Richard T. Silver*, CA-07968); Rhode Island Hospital, Providence (*Louis A. Leone*, CA-08025); Roswell Park Memorial Institute, Buffalo, NY (*Leon Stutzman*, CA-02599); SUNY Affiliated Hospitals (Downstate), Brooklyn, NY (*Fred Rosner*, CA-12886); and West Virginia University Medical Center, Morgantown (*John B. Harley*, CA-07757). Eastern Cooperative Oncology Group—Albany Medical College, NY (*John Horton*, CA-06594); Boston University, Mass (*Albert Schilling*, CA-11109); Georgetown University School of Medicine, Washington, DC (*Bruce I. Shnider*, CA-02824); Jackson Memorial Hospital, Miami, Fla (*Jacob Colsky*, CA-02822); Johns Hopkins

BCNU has been shown to have marked antileukemic (1–6) and antitumor activities in experimental animals (7) and in clinical trials (8–10). The present randomized study was designed jointly by members of the Acute Leukemia Group B and the Eastern Cooperative Oncology Group to determine the effectiveness of BCNU in the treatment of Hodgkin's disease and lymphomas, and also to obtain toxicologic data on this drug. Two dose levels were used for each of the two schedules of administration.

MATERIALS AND METHODS

Patient Selection

All patients considered for this study had a diagnosis of Hodgkin's disease or lymphoma (including lymphocytic lymphosarcoma, lymphoblastic lymphosarcoma, and reticulum cell sarcoma, but not giant follicle lymphoma or reticulum cell sarcoma of the bone) as established by a biopsy. In addition, each patient had: (*a*) no significant lymphocytic infiltration of the bone marrow as evidenced by bone marrow biopsy or as reflected by a peripheral white blood cell count (wbc) over 15,000 cells/mm^3 with over 50% lymphocytes; (*b*) more than one area of tumor involvement and either stage III or IV disease; (*c*) tumor masses that were measurable either by calipers or on roentgenograms; (*d*) adequate bone marrow function as indicated by a total wbc of 4000 cells/mm^3 and a platelet count of over 100,000 platelets/mm^3; (*e*) adequate renal function as indicated by a blood urea nitrogen (BUN) value of 25 mg/100 ml or less or a nonprotein nitrogen (NPN) value of 40 mg/100 ml or less; (*f*) no chemotherapy, extensive irradiation, or high-dose corticosteroid therapy for 2 weeks prior to entry into the study; and (*g*) an expected survival of more than 2 months.

Hospital, Baltimore, Md (*Raymond E. Lenhard, Jr.*, CA-06973); Maimonides Medical Center, Brooklyn, NY (*John H. Edmonson*, CA-05588); and NCI Cancer Research Center, Baltimore, Md (*Arthur A. Serpick*).
Supported by Public Health Service grants (CA) from the NCI, National Institutes of Health, Department of Health, Education, and Welfare.

Mechanics of Study

Those patients who fulfilled the criteria were randomly assigned to one of the four regimens of treatment on the BCNU protocol as shown in the schema of table 1. The initial evaluation included: history, physical examination, laboratory studies (hemoglobin or hematocrit values, leukocyte [total and differential] counts, platelet count, BUN or NPN, uric acid, alkaline phosphatase, serum glutamic oxaloacetic transaminase (SGOT), total serum protein, albumin-globulin ratio, serum protein electrophoretic determinations), and chest roentgenograms. During therapy, measurements of palpable lesions, determinations of hemoglobin or hematocrit values, and leukocyte or platelet counts were done weekly; roentgenograms (for lesions observed by these studies), BUN or NPN, alkaline phosphatase, and SGOT determinations were done every 2 weeks.

BCNU was supplied as a lyophilized powder in 100-mg vials. A separate 3-ml vial of 50% alcohol was supplied as a diluent. For use, 100 mg of BCNU was dissolved in the diluent. After further dilution with 5 ml of normal saline, the compound was added to an infusion bottle of 5% glucose/water and the dose was administered intravenously (iv) over a 30-minute period. Drug modification and hematologic toxicity, as outlined in table 2, served as guidelines for reduction in the BCNU dose level.

Patients who achieved remission (complete or partial) by Day 41 of the study became eligible for maintenance treatment with BCNU; the investigator determined the dose level through analysis of the initial dose response. Those who failed to achieve remission (no change or progression) by Day 41 of the study were considered to be off-study.

Definitions of responses which included complete regression (CR), partial regression (PR), no change (NC), and progression (P) are as outlined in a previous group study (11). Patients who had a CR or PR were considered to be responders, and those who had NC or P were considered to be nonresponders. Adequate trial included treatment which produced toxicity and which, in the absence of response,

TABLE 1.—BCNU protocol schema

Randomized regimens	Induction phase	Response	Maintenance phase
I	Single high: 250 mg/m², iv single dose	CR or PR	Maintenance dose determined by the investigator based on analysis of initial dose response
II	Single low: 168 mg/m², iv single dose	CR or PR	Maintenance treatment until relapse, then off-study
III	Weekly high: 90 mg/m², iv weekly × 6	NC or P	Off-study
IV	Weekly low: 60 mg/m², iv weekly × 6	NC or P	Off-study

TABLE 2.—Hematologic toxicity score and drug modification

Toxicity score	Peripheral blood cell counts		Drug dose
	Wbc (cells/mm³)	Platelets (cells/mm³)	
0 = None	≥5000	≥100,000	Full dose
1 + = Mild	<5000–4000	≥100,000	Half dose
2 + = Moderate	<4000–3500	<100,000–75,000	Half dose
3 + = Severe	<3500	< 75,000	No drug
4 + = Life-threatening or fatal	Same counts as for 3+ toxicity plus infection, bleeding, etc		No drug

was continued so that the duration of drug administration and subsequent toxicity was at least 42 days (or, in the case of the single-injection regimen, the patient was observed for that duration). Patients whose objective tumor measurements increased to at least 125% of the initial sum of the products of the diameters after 28 days of treatment and subsequent toxicity were also considered to have had an adequate trial.

RESULTS

Sixty-eight patients were entered into the study program between January 1965 and May 1967. Twenty-four patients were invalidated from the study protocol for the following major reasons: (*a*) not eligible for the protocol because of diagnostic deviation or because of lack of measurable disease (ten patients); (*b*) improper dose level, or concomitant therapy with prednisone[7] or extensive radiation (eight patients); (*c*) inadequate trial (six patients).

Forty-four patients were considered valid. The ages of these patients ranged from 19 to 78 years. There were 30 men and 14 women.

[7] Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

TABLE 3.—Response by dose and schedule

Dose regimen	Total No. of patients	No. of patients responding			
		CR	PR	NC	P
Single high	14	3	2	4	5
Single low	10	2	4	2	2
Weekly high	10	0	5	1	4
Weekly low	10	0	3	2	5
Total	44	5	14	9	16

Thirty-three patients had a diagnosis of Hodgkin's disease, seven had lymphosarcoma, and four had reticulum cell sarcoma.

There were a total of 19 objective responses, five complete and 14 partial, with an overall response rate of 43% (19 of 44) (table 3). The probability of response (CR and PR) did not vary significantly by dose or schedule. Among all the responders, however, the chance of producing a CR was significantly greater in the single-injection regimen than in the weekly injection regimen, with no regard for dose ($P = 0.039$).

As shown in table 4, the duration of response varied from 40 to 771 days. It is of interest (but not statistically significant) that the two long-term responders were in the single-injection group.

TABLE 4.—Duration of response in days

Dose regimen	Duration of response (days) for patients responding	CR + PR (%)
Single high (5 patients)	57, 74, 85, 92, 738	36
Single low (6 patients)	51, 56, 76, 148, 182, 771	60
Weekly high (5 patients)	40, 72, 81, 84, 144	50
Weekly low (3 patients)	56, 57, 59	30

The response rate was 48% for Hodgkin's disease (16 of 33 patients, four CRs) and 27% for the lymphomas (three of 11 patients, no CRs) (table 5).

TOXICITY

The various toxic reactions and their severity are recorded in table 6. The toxic reactions observed most often were bone marrow depression, nausea, and vomiting. Thirty-four patients encountered depression of at least one of the formed elements of the blood. Thirty-one patients showed leukocytic toxicity, and 24 showed platelet toxicity. In two patients the toxicity was severe and resulted in death.

For the various regimens, the median nadirs of the wbc were between 1.9 and 3.1 × 10^3 cells/mm^3, and they occurred on medians between 30 and 43 days after the start of treatment (table 7). The median numbers of days to recover from the wbc nadirs were between 11 and 16 days. There was no remark-

TABLE 5.—Distribution of diagnosis and response by treatment regimen*

Disease	Single high		Single low		Weekly high		Weekly low		Total	
	R	NR	R	NR	R	NR	R	NR	R	NR
Hodgkin's disease	5	4	5	2	3	5	3	6	16	17
Lymphosarcoma	0	3	1	2	1	0	0	0	2	5
Reticulum cell sarcoma	0	2	0	0	1	0	0	1	1	3
	5	9	6	4	5	5	3	7	19	25
Total No. of patients	14		10		10		10		44	

*R = responders; NR = nonresponders.

TABLE 6.—Severity scoring of toxic effects

Type of toxicity*	No. of patients showing indicated degree of toxicity while receiving treatment regimen—				
	Single high	Single low	Weekly high	Weekly low	Totals
Hematologic score					
0	3	1	2	4	10
1	1	1	0	1	3
2	1	1	0	0	2
3	7	7	8	5	27
4	2	0	0	0	2
Gastrointestinal score					
0	8	6	4	3	21
1	4	3	6	6	19
2	2	1	0	1	4
3	0	0	0	0	0
4	0	0	0	0	0
Other score (renal, hepatic, etc)					
0	10	9	7	9	35
1	4	1	3	1	9
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
Total No. of evaluable patients	14	10	10	10	44

*Toxicity score: 0 = none; 1 = mild; 2 = moderate; 3 = severe; 4 = life-threatening or fatal.

able difference in the depression by dose or schedule except that depression occurred somewhat less frequently among those patients in the weekly low-dose group. When it did occur, however, it appeared to be just as severe and lasted just as long as in the other regimens. The single-high-dose group had the highest frequency of failure to recover from the wbc toxicity; the two toxic deaths were in this group.

The median nadirs of the platelet count for the various regimens were between 28,000 and 51,000 platelets/mm^3 and occurred on medians between 24 and 42 days after the start of treatment (table 8). The median numbers of days to recover from the platelet count nadirs were between 7 and 22 days. The maximum platelet count depression occurred later on the weekly injection schedule than on the single-injection schedule but was of nearly equal severity and probably required just as long for recovery.

Nausea and vomiting occurred in 19 patients during or a few hours after the administration of BCNU. They were more prolonged in four patients, lasting up to 24 hours, but in all patients they were controlled with antiemetics.

Only one patient showed a progressive elevation of BUN, bilirubin, and alkaline phosphatase values. This patient also had other evidence of progressive disease which might account for the azotemia and abnormal liver function. Five other patients had abnormal liver function test results (bilirubin, alkaline phosphatase, SGOT, and lactic dehydrogenase) while receiving therapy.

Transient local venous pain occurred in one patient, and in another patient there was transient burning at the injection site with a generalized itching and flushing of the face.

At the time of evaluation, five deaths had occurred either during or after drug administration. These cases were carefully reviewed, and based upon the investigator's

TABLE 7.—Hematologic (wbc) toxicity

	Single high	Single low	Weekly high	Weekly low
		Nadir of wbc (cells × 10³/mm³)		
Minimum	0.3	1.3	1.6	0.4
Median	2.2	1.9	3.1	3.1
Maximum	4.3	4.7	3.9	4.5
No. of patients with depression <5.0	10	8*	8	5
No. of patients with no depression <5.0	4	2	2	5
		Day of wbc nadir		
Earliest	20	7	33	21
Median	30	43	43	39
Latest	57	52	68	56
		No. of days to recovery from wbc nadir		
Shortest	7	3	2	7
Median	16	14	11	16
Longest	35	24	41	27
No. of patients with no recovery before going off-study	4	1	2	1

*In one patient toxicity was difficult to gauge.

TABLE 8.—Hematologic (platelet) toxicity

	Single high	Single low	Weekly high	Weekly low
		Nadir of platelet count (cells × 10³/mm³)		
Minimum	0	29	29	18
Median	28	51	46	50
Maximum	73	82	80	92
No. of patients with depression <100	9	5	6	4
No. of patients with no depression <100	5	5	4	6
		Day of platelet count nadir		
Earliest	19	21	40	32
Median	27	24	42	37
Latest	57	38	93	50
		No. of days to recovery from platelet count nadir		
Shortest	5	3	7	3
Median	18	12	22	7
Longest	31	39	43	11
No. of patients with no recovery before going off-study	3	0	0	1

opinion and the available postmortem examination reports two of the deaths were considered to be related to drug toxicity. Both of these patients had a diagnosis of reticulum cell sarcoma and were receiving treatment according to the single-high-dose regimen.

Patient No. 1, a 49-year-old woman with reticulum cell sarcoma, received a single 370-mg dose of BCNU. From a total wbc of 8100 cells/mm³ on Day 1, the count dropped to 450 cells/mm³ on Day 26; the patient died on Day 27 with infection. A postmortem examination was not performed, but the death was considered to be directly related to drug toxicity.

Patient No. 2, a 38-year-old man who also had reticulum cell sarcoma, received a single 400-mg dose of BCNU. This patient had evidence of bone marrow invasion by tumor prior to the start of therapy with BCNU. He was treated with corticosteroids because of his clinical deterioration even though his peripheral blood cell counts were normal. On Day 1, this patient had wbc and platelet counts of 6100 and 510,000 cells/mm³, respectively. By Day 20, the platelet count had dropped to 30,000 platelets/mm³, and on Day 27 it had dropped further to 0 platelets/mm³ with a wbc of 800 cells/mm³. The patient died on Day 28. Postmortem examination revealed (*a*) extensive malignant lymphomatous involvement of various organs including the bone marrow; (*b*) hemorrhage of the stomach due to lymphoma but complicated by glucocorticoid therapy and thrombocytopenia; and (*c*) icterus secondary to hepatomegaly with lymphomatous involvement, possibly due to BCNU therapy. In this case, death was considered to be primarily the result of progressive disease, although BCNU treatment was felt to have contributed to the fatal outcome.

Efforts were made to correlate hematologic toxicity with response (table 9): seven of 19 patients (37%) responded in the absence of platelet count depression below 100,000 platelets/mm³ but had a median drop in platelet count of 160,000 platelets/mm³; twelve of 23 (52%) responded who had a platelet count depression to below 100,000 platelets/mm³ and a median drop in platelet count of 195,000 platelets/mm³. There was no indication that wbc depression by itself was associated with an increased likelihood of response.

In respect to nearly every characteristic examined, the 19 responders and the 25 nonresponders were remarkably similar. As shown in table 10, there was no indication that they differed in age, duration of disease prior to this study, drugs received previously, response to these drugs, amount of BCNU received, or frequency of different diagnoses.

TABLE 9.—Hematologic toxicity and response

Platelet depression ($\times 10^3$ cells/mm³)	Wbc depression ($\times 10^3$ cells/mm³)	No. of patients*	No. of responding patients	No. of nonresponding patients	Response rate (%)
None	None	9	4	5	44
None	<5.0	10	3	7	30
<100	None	3	0	3	0
50–99	<5.0	6	3	3	50
<50	<5.0	14	9	5	64
Total		42	19	23	45

*Two cases were not evaluable for maximum depression of peripheral blood elements and have been excluded from this table. Of the 42 evaluable patients, 30 patients had wbc $<5 \times 10^3$ cells/mm³ (15 responders, 50% response rate) and 12 patients had no wbc depression (4 responders, 33% response rate). Twenty-three of 42 patients had platelet counts $<100 \times 10^3$ cells/mm³ (12 responders, 52% response rate) and 19 patients had no platelet count depression (7 responders, 37% response rate).

TABLE 10.—Comparability of responders and nonresponders

Characteristics	Responders (19 patients)	Nonresponders (25 patients)
Men	74%	64%
Women	26%	36%
Age (yrs):		
Youngest	21	19
Median	33	42
Oldest	78	65
Intervals (mos) from diagnosis to BCNU study:		
Shortest	4	2
Median	26	29
Longest	118	192
No previous response	32%	20%
Three or more previous responses	32%	36%
Total BCNU (mg):		
Least	245	200
Median	385	425
Maximum	1190	960
Measured tumors:		
Nodes only	33%	48%
Nodes + spleen only	39%	20%
Other	28%	32%

DISCUSSION

If usefulness of a drug in the experimental animal system (1,4,7) is any consideration, BCNU is an effective drug. In clinical trials DeVita et al (8) have shown an overall response of 21% in a wide variety of malignant conditions. Lessner (9), of the Southeastern Cancer Chemotherapy Cooperative Study Group, has reported an overall response rate of 55% (17 of 31 patients) and Young et al (10) reported 47% (21 of 45 patients) in advanced Hodgkin's disease treated with BCNU. In the present study, the overall response rate for Hodgkin's disease and the lymphomas was 43% (19 of 44 patients). The patients with Hodgkin's disease appeared to respond better (16 of 33 patients) than those with the lymphomas (three of 11 patients); however, the latter group was so small that no precise statements could be made. If one considers the fact that these patients had extensive prior treatment with chemotherapy and in some cases with radiotherapy as well, then the clinical experience with BCNU appears to be quite encouraging.

BCNU has been shown to have a high therapeutic ratio reflecting the activity over a wide range of doses (4). Also, it was effective regardless of the treatment schedule with evidence of increased survival on various schedules (4). Similar observations were made in the current study where the probability of response (CR or PR) did not vary significantly by dose or schedule.

Patients who had failed to respond to prior therapy were as likely to respond to BCNU as those patients who had demonstrated responsiveness previously (table 11). Also, the responses to each of the several agents given previously were similar for BCNU responders and nonresponders.

Toxicity

Hepatic and renal dysfunction were slight and the only prolonged and major toxic effects

were related to the hematopoietic system. Delayed toxicity (as noted by others) with a fall in the leukocyte and platelet counts in the peripheral blood was observed between 4 and 6 weeks after the start of therapy. In most cases this was reversible, with recovery in the wbc and platelet count occurring between 1 and 3 weeks after the counts had reached their nadir.

The cause of delayed toxicity is not clear, but Tsukagoshi et al (12) have shown a decrease in the activity of nicotinamide adenine dinucleotide glycohydrolase (NADase) in the various organs of mice treated with BCNU. After an initial increase there was a fall in the NADase activity of the spleen, reaching a nadir in 8–11 days after the start of therapy and followed by a slow recovery. The authors suggest a similarity between the delayed and prolonged toxic effects of BCNU observed in clinical studies by others and the delayed and prolonged suppression of NADase activity by BCNU noted by them in animal studies.

The similarity of the biologic effects of BCNU to those of the accepted alkylating agents has led to its inclusion in this class of agents (13–16). This inclusion is now questioned by many (4,17,18) on the basis of laboratory studies. In our clinical studies, the response to BCNU did not appear to be related to the response to prior alkylating agents, mechlorethamine and cyclophosphamide.[8] Somewhat similar conclusions were drawn by Lessner (9).

Of the nine patients initially sensitive (responders) to cyclophosphamide and the 18 sensitive to mechlorethamine (who then became resistant to these agents), four and six patients, respectively, showed subsequent sensitivity to BCNU. Also, of the 13 nonresponders to cyclophosphamide and the six nonresponders to mechlorethamine, five and four, respectively, showed a subsequent

TABLE 12.—Distribution of responses to BCNU and prior alkylating agents*

Treatment	R	NR
Cyclophosphamide (22 patients)	9	13
Mechlorethamine (24 patients)	18	6
Subsequent BCNU treatment		
Cyclophosphamide R (9 patients)	4	5
Cyclophosphamide NR (13 patients)	5	8
Mechlorethamine R (18 patients)	6	12
Mechlorethamine NR (6 patients)	4	2

*R = responders, CR or PR; NR = nonresponders, NC or P.

[8] Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; HN2; nitrogen mustard.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino] tetrahydro-, 2-oxide, monohydrate; Cytoxan.

TABLE 11.—Response to BCNU as related to response to previous drugs

Previous treatment	BCNU responders			BCNU nonresponders		
	No. treated (%)	No. of responses	Response rate (%)	No. treated (%)	No. of responses	Response rate (%)
Vincristine	5 (26)	4	80	8 (32)	3	38
Vinblastine*	13 (68)	8	62	15 (60)	10	67
Cyclophosphamide	9 (47)	3	33	13 (52)	6	46
Mechlorethamine	10 (53)	6	60	14 (56)	12	86
Procarbazine	3 (16)	1	33	2 (8)	1	50
Steroids	3 (16)	1	33	5 (20)	3	60
X-ray	9 (47)	8	89	11 (44)	9	82
Other drugs	2 (11)	1	50	4 (16)	2	50

* Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate.

sensitivity to BCNU. Interestingly, the quality of therapeutic response to BCNU in some cases was also superior to that of the prior alkylating agent responses. In view of all of this, one wonders if alkylation is the sole mode of action of BCNU.

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Phase I Clinical Trial of a Single-Dose and Two Weekly Schedules of ICRF-159 (NSC-129943) [1,2]

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SUMMARY

Initial clinical (phase I) evaluation of ICRF-159, an oral antineoplastic agent of novel structure, was carried out in 38 patients with advanced neoplastic disease using single doses and two weekly schedules. Single doses of 1.0–10.5 g/m² were given and resulted in only mild and inconsistent hematologic toxicity at all doses above 1.0 g/m². A weekly schedule of 3.0 g/m² as a single weekly dose × 6 (schedule I) produced only mild to moderate leukopenia. A dose of 3.0 g/m² given as 1.0 g/m² every 6 hours × 3 once weekly × 6 (schedule II) produced moderate to marked leukopenia. No important toxicity other than leukopenia was seen. Schedule II is recommended for phase II trial in patients who have not had extensive prior chemotherapy.

[Cancer Chemother Rep 58:393–400, 1974]

ICRF-159 is a synthetic antineoplastic agent developed by Creighton, Hellmann, and Whitecross (1) as a nonpolar derivative of the complexing agent ethylenediaminetetraacetic acid. Its structure is shown in figure 1. It produces a greater than 100% increase in lifespan against L1210 leukemia in mice on a Day 1, 5, and 9 treatment schedule, which is the optimal schedule of administration (2). It also prevents the development of pulmonary metastases in animals with subcutaneously implanted Lewis lung tumor (3). It shows a wide spectrum of antitumor activity against various animal tumors[6] and has been used clinically both alone and in combination (4,5). We report the tolerance to three dosage regimens: (*a*) single doses of from 1.0 to 10.5 g/m² of body surface area, (*b*) weekly doses starting at 3.0 g/m², and (*c*) weekly doses starting at 3.0 g/m² given in three divided doses at 6-hour intervals.

Materials and Methods

Patients

Patients with histologically confirmed cancer not amenable to conventional treatment were eligible for inclusion in the study provided their renal function was adequate as determined by a blood urea nitrogen (BUN) level of less than

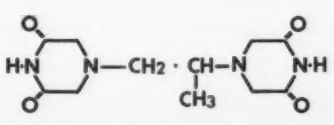

FIGURE 1.—Structure of ICRF-159.

[1] Received Sept 24, 1973; revised Dec 20, 1973; accepted Jan 14, 1974.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text. ICRF-159 was supplied by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute (NCI).

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[6] Friedman MA, and Carter SK. Clinical brochure: ICRF 159 (NSC-129943). Prepared by the NCI, Bethesda, Md, 1970.

25 mg/100 ml and a serum creatinine level of less than 1.5 mg/100 ml. Other requirements included a serum bilirubin level of less than 1.5 mg/100 ml, a serum glutamic oxaloacetic transaminase (SGOT) level of less than 100 IU, an absence of gross edema, pretreatment white blood cell (wbc) and platelet counts in excess of 5000 and 100,000 cells/mm^3 respectively, and a sufficient interval after previous chemotherapy to avoid possible additive toxicity (2–8 weeks, depending on the preceding treatment). Patients receiving nontoxic doses of ICRF-159 were eligible for a subsequent course at the next level after an interval of at least 2 weeks. Patients who had toxic reactions were not reentered into the study with the following exceptions: patients who received single doses and had mild leukopenia (wbc $> 4.0 \times 10^3$ cells/mm^3, normal platelet count) subsequently received larger single doses after the clearing of all toxicity; all patients who received 10.5 g/m^2 as a single dose subsequently received 3.0 g/m^2 as a single dose weekly starting not less than 3 weeks after the single dose and after the clearing of all toxicity. One patient who had mild leukopenia (wbc $> 4.0 \times 10^3$ cells/mm^3, normal platelet count) and had received treatment weekly according to schedule I (*see* below) subsequently received weekly schedule II treatment, and one patient who received weekly schedule I and had no toxicity subsequently received weekly schedule II. In both cases, an interval of 3 weeks separated the two weekly schedules.

Determinations of wbc, differential leukocyte, platelet, and reticulocyte counts were performed twice weekly during treatment and for at least 3 weeks after the completion of each treatment course. Urinalysis and determinations of hematocrit, BUN, serum creatinine, SGOT, alkaline phosphatase, bilirubin, total serum proteins, serum albumin, and serum calcium values were performed once weekly. Measurable tumors were measured by caliper using the product of the greatest horizontal and vertical diameters. Additional examinations such as skeletal and chest roentgenograms were obtained before the study. The chest roentgenograms were repeated every 2 weeks whenever measurable tumors were present; for patients without measurable tumors, roentgenographic examinations were repeated at the end of each study or when indicated clinically. The patients treated, their diagnoses, and their prior treatment are shown in table 1.

Drug Administration

ICRF-159 was supplied as tablets of 50 and 250 mg. They were stored at room temperature and given orally. Patients receiving the drug according to schedule I received 3.0 g/m^2 as a single dose on Day 1. This dose was repeated weekly for 6 weeks in the absence of toxicity. In the presence of a wbc less than 5000 but greater than 3000 cells/mm^3 and/or a platelet count less than 100,000 but greater than 75,000 cells/mm^3 the dose was reduced to 2.0 g/m^2/week until both hematologic parameters had recovered. In the presence of a wbc less than 3000 cells/mm^3 or a platelet count less than 75,000 cells/mm^3 treatment was stopped until both counts rose above these levels. Patients with a wbc less than 1000 cells/mm^3 received no further ICRF-159. Patients treated according to schedule II received 3.0 g/m^2 on Day 1 divided into three equal doses given 6 hours apart. This was repeated weekly in the absence of toxicity. In the presence of hematologic toxicity the same dose reduction was used as for schedule I. Patients were followed for 3 weeks after the end of treatment. A detailed study protocol is available upon request.

RESULTS

Single-Dose Treatment

Thirty patients received 35 courses of ICRF-159 as a single dose. One patient (No. 35) had vomiting for 4 days after treatment (3.3 g/m^2) and one patient (No. 2) had very slight alopecia after 7.9 g/m^2. Apart from this, the only toxicity noted was hematologic (table 2). Toxicity was principally leukopenia, which occurred erratically at all doses greater than 1.0 g/m^2, was mild (a wbc < 3000 cells/mm^3 was not recorded), and showed essentially no dose response. Thrombocytopenia occurred in only two patients and one of these (No. 23), who received 5.9 g/m^2 and had thrombocytopenia to 64,000 cells/mm^3, was a chronic alcoholic who subsequently had thrombocytopenia of 36,000 cells/mm^3 in the absence of any drug therapy.

TABLE 1.—Diagnoses and prior therapy in patients treated with ICRF-159

Patient No.	Age (yrs)	Diagnosis	Previous treatment*
1	48	Epidermoid carcinoma, lung	R, cyclophosphamide
2	62	Epidermoid carcinoma, lung	R, isophosphamide
3	68	Epidermoid carcinoma, lung	R
4	59	Epidermoid carcinoma, lung	R, mechlorethamine, methotrexate, CCNU, hydroxyurea
5	47	Adenocarcinoma, lung	R, streptozotocin, bleomycin
6	42	Adenocarcinoma, lung	Isophosphamide
7	46	Anaplastic large cell carcinoma, lung	S, R
8	34	Anaplastic large cell carcinoma, lung	R, vincristine, hydroxyurea, CCNU, procarbazine, NSC-45388
9	61	Anaplastic large cell carcinoma, lung	CCNU, NSC-141540
10	56	Anaplastic large cell carcinoma, lung	R
11	72	Adenocarcinoma, colon	S, 5-FU
12	69	Adenocarcinoma, colon	S
13	77	Adenocarcinoma, colon	5-FU
14	62	Adenocarcinoma, rectum	S, R
15	55	Adenocarcinoma, rectum	R, 5-FU, cyclophosphamide
16	47	Adenocarcinoma, stomach	S, isophosphamide
17	60	Adenocarcinoma, stomach	S, camptothecin, isophosphamide
18	55	Adenocarcinoma, stomach	5-FU
19	49	Epidermoid carcinoma, esophagus	S, R
20	60	Epidermoid carcinoma, esophagus	Streptozotocin, bleomycin
21	48	Adenocarcinoma, breast	S, R, 5-FU
22	42	Adenocarcinoma, breast	S, R, mechlorethamine, 5-FU
23	54	Epidermoid carcinoma, head and neck	Isophosphamide
24	51	Epidermoid carcinoma, head and neck	—
25	63	Adenocarcinoma, salivary gland	S, R

TABLE 1.—Diagnoses and prior therapy in patients treated with ICRF-159—Continued

Patient No.	Age (yrs)	Diagnosis	Previous treatment*
26	55	Clear cell carcinoma, kidney	R, CCNU, streptozotocin, bleomycin
27	49	Clear cell carcinoma, kidney	—
28	46	Malignant adnexal tumor, finger	S, 5-FU, bleomycin
29	78	Adenocarcinoma, pancreas	S, isophosphamide
30	51	Transitional cell carcinoma, bladder	—
31	55	Class V sputum cytology	—
32	52	Anaplastic carcinoma, primary site unknown	S, R, camptothecin, bleomycin, CCNU, methotrexate
33	83	Anaplastic carcinoma, primary site unknown	R, NSC-122819, camptothecin
34	49	Anaplastic carcinoma, primary site unknown	NSC-141540
35	36	Synovial sarcoma, knee	S, cyclophosphamide, 5-FU, bleomycin, methotrexate
36	57	Fibrosarcoma, retroperitoneum	S, isophosphamide, adriamycin, cyclophosphamide, methotrexate, actinomycin D
37	48	Fibrosarcoma, back	Adriamycin
38	54	Fibrosarcoma, back	S

*S = surgery; R = radiotherapy.

Since there was no dose response, all patients receiving doses greater than or equal to 2.0 g/m² have been pooled for the following analysis. The mean overall decrease in wbc was 32%. There was virtually no differential effect on granulocytes (73% pretreatment, 68% at the nadir of wbc). The decrease in platelets was 22%. The median time to nadir was 12 days in patients with leukopenia. The reticulocyte count decreased from a mean of 1.4% to a mean of 0.6%. Median nadir of reticulocyte count was 5 days (range, 4–8 days). A fall of more than 5 percentage points in the hematocrit value was seen in four patients. No change was seen in BUN, serum creatinine, serum bilirubin, SGOT, serum alkaline phosphatase, or serum calcium levels.

Weekly Dosage Treatment

Six patients received ICRF-159 weekly on schedule I and ten on schedule II. Leukopenia and thrombocytopenia were the only drug toxic effects seen. All patients except one (No. 6) had toxic reactions to the weekly dosage. This patient did not have toxic reactions to either weekly treatment schedule. Toxicity was milder, took longer to develop, and required more drug to become apparent on schedule I than on schedule II (table 3, fig 2). Two patients receiving schedule II (Nos. 4 and 8) had pronounced

TABLE 2.—Hematologic toxicity after single doses of ICRF-159

Dose (g/m^2)	No. of patients treated	Wbc count: No. of patients with leukopenia	Wbc count: Nadir ($\times 10^3$ cells/mm^3)	Wbc count: No. of days to nadir (mean)	Platelet count: No. of patients with thrombocytopenia	Platelet count: Nadir ($\times 10^3$ cells/mm^3)	Platelet count: No. of days to nadir
1.0	3	0	—	—	0	—	—
2.0	2	1	4.3	12	0	—	—
3.3	5	3	4.4 (3.8–4.8)	12	0	—	—
4.4	3	2	3.2, 4.3	5, 11	0	—	—
5.9	5	3	4.6 (4.3–4.8)	12 (11–13)	1	64	13
7.9	8	5	4.1 (3.3–4.9)	15 (11–18)	1	97	11
10.5	9	3	3.6 (3.3–3.9)	13 (11–15)	0	—	—

toxic reactions (wbc 700 cells/mm^3 and platelet count 64,000 cells/mm^3 in patient No. 8; wbc 800 cells/mm^3 and platelet count 21,000 cells/mm^3 in patient No. 4). Both patients had had extensive prior treatment (table 1). Counts returned promptly to normal on cessation of treatment and were readily controlled by adjustment of dosage (fig 2). No drug-related deaths occurred. No objective tumor responses were seen; however, most patients had advanced disease which was not measurable and many had had extensive prior treatment.

DISCUSSION

ICRF-159 is an antineoplastic agent of novel structure. It is active in early mitosis (6) and inhibits DNA synthesis (7) by a mechanism which is so far unknown, though it has been suggested on the basis of in vitro studies that it might acylate biological nucleophiles (7). It has shown activity in patients with acute leukemia (8) but has shown little activity so far as a single agent against low growth fraction tumors. However, in experimental systems it has demonstrated the interesting and potentially important effect of protecting against the cardiac toxicity of the anthracycline antibiotics (9). Its use in man has so far been largely on a divided daily dose schedule [7] and since it appears from a number of aspects to be a drug worthy of further study both alone and in combination we chose to define its toxicity in weekly doses. Single-dose toxicity was evaluated before weekly doses were administered, to define more precisely the kinetics of toxicity and its quantitative relationship to dosage. From the reports of British data (10) the minimum toxic dose in man appeared to be in the order of 3.0 g/m^2, and therefore we chose

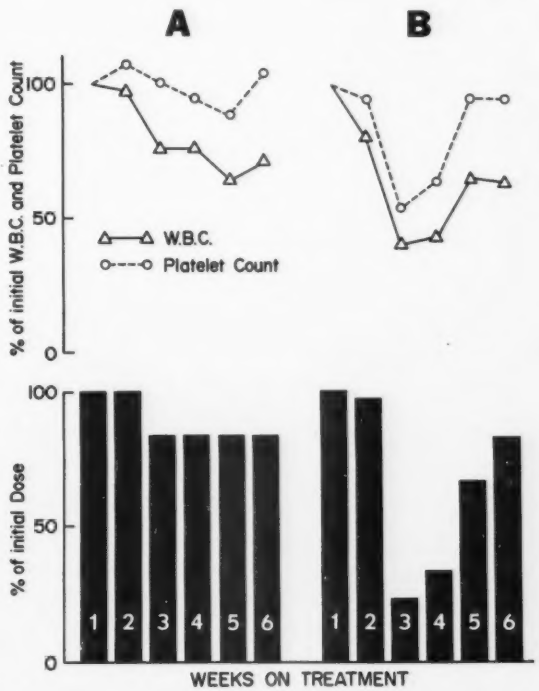

FIGURE 2.—Wbc and platelet counts in patients receiving ICRF-159 weekly on schedule I (*A*) and schedule II (*B*).

[7] *See* footnote 6.

TABLE 3.—Hematologic toxicity after weekly doses of ICRF-159

Schedule	No. of patients treated	No. of patients with wbc count—(cells/mm³)				Wks to wbc toxicity*		Wbc nadir (× 10³/mm³)		No. of patients with platelet count—(cells/mm³)		
		> 5000	3000–4900	1000–2900	< 1000	Median	Range	Median	Range	> 100,000	99,000–75,000	< 74,000
I	6	1	4	1	0	3	2–6	3.8	2.7–4.9	6	0	0
II	10	1	1	6	2	2	1–2	2.1	0.7–3.9	7	1	2

*The patient who did not have hematologic toxicity is not included in these figures.

one third of this dose as a starting dose and escalated it as indicated in table 4. Dose-limiting toxicity, however, was not seen at any dose up to 10.5 g/m² nor did there seem to be a trend in the direction of more marked or more consistent toxicity. The single-dose trial was therefore terminated after the nature and kinetics of toxicity had been determined. From the results of the single-dose trial it appeared that intestinal absorption might be limited at doses above 3.0 g/m². We therefore investigated a single and a divided weekly dose starting at about one third of the highest dose given as a single dose. It did not prove necessary to escalate the dose. Dose-limiting toxicity consisting of leukopenia was achieved in nine of ten patients treated with schedule II. Marked thrombocytopenia in the absence of dose-limiting leukopenia did not occur and no other toxicity was noted. The schedule proved unacceptably toxic in only two patients who had received extensive prior treatment. The toxicity was easily manageable. Schedule I produced toxicity which was neither as consistent nor as profound as the toxicity of schedule II. No absorption studies in animals were available when the present trial was initiated. However, we have reported (11) that at doses greater than 3.0 g/m² in man the total absorption of drug is essentially independent of the dose level, and absorption is more than 100% greater on schedule II than on schedule I, confirming the impression reached clinically that poor absorption may account at least in part for the schedule dependence.

While the present report was in preparation, the results of a study on ICRF-159 given in divided doses every 8 hours for 3 days were reported (12). The maximum tolerated dose was 1.0 g/m²/day. This is in good agreement with the concepts of marked schedule dependence noted here.

From the present studies we conclude that ICRF-159 may safely be given in phase II initial therapeutic trials using schedule II (3.0 g/m² in three divided doses [6 hours apart] once weekly) except in patients who have had extensive prior chemotherapy. In these patients, an initial trial of schedule I might be used to test susceptibility to the drug. From these results it would appear that a split schedule consisting of more than three divided doses might well prove unacceptably hazardous in some patients.

Finally, it must be clear from these data that clinical trials using an orally administered drug without adequate data on gastrointestinal absorption, even when the drug has been extensively used clinically in small individual doses, can be time consuming and costly.

LIST OF COMPOUNDS

Adriamycin: NSC-123127
Actinomycin D: NSC-3053; dactinomycin
Bleomycin: NSC-125066
Camptothecin: NSC-100880; CAS reg. No. 25387-67-1
CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU
Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1
Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide
Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; HN2; nitrogen mustard
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl)-methylamino]benzoyl]-
NSC-45388: CAS reg. No. 4342-03-4; 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; DTIC
NSC-122819: 4'-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside); VM 26
NSC-141540: 4'-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); VP-16-213
Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride
Streptozotocin: NSC-85998
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Total Body Radiation Therapy for Disseminated Lymphosarcoma: Results of a Pilot Study [1,2]

K. Brace, [3] M. J. O'Connell, [4] V. Vogel, [4] and A. Schantz [5,6]

SUMMARY

Nine patients with disseminated lymphosarcoma were treated with total body radiation (TBR) using a fractionated treatment regimen. Seven of these patients (six previously untreated) with generalized nodal disease and bone marrow infiltration received TBR alone. The other two patients (one previously untreated) had generalized nodal disease and were treated with TBR followed by total nodal radiation. The radiation therapy was uniformly well tolerated with no serious gastrointestinal or hematologic toxic effects. Three of the patients had complete remission (2, 7, and 37+ months in duration), four patients had partial regression of adenopathy with control of the disease process (1, 4, 5, and 11 months in duration), and two patients had no objective response. The eight patients with persistent or recurrent disease were treated with chemotherapy but tolerated treatment poorly, requiring delays in therapy, decreases in dosage, and/or premature discontinuation of therapy due to inadequate bone marrow reserves. Although TBR was well tolerated and produced objective disease response in seven of nine patients treated, the duration of response was relatively short and the ability to subsequently deliver effective combination chemotherapy was seriously compromised.

[Cancer Chemother Rep 58:401–405, 1974]

Total body radiation therapy (TBR) has been used for the treatment of a variety of neoplastic disorders for over 40 years (1–10).[7] The most encouraging results have been observed in patients with chronic lymphatic leukemia (6) and disseminated lymphosarcoma (4,5,7–10).

In view of the favorable preliminary experience with TBR in the management of advanced lymphosarcoma reported by others (4,5,7), a pilot study was initiated at the Baltimore Cancer Research Center, Division of Cancer Treatment, National Cancer Institute, in 1970 to further investigate the value of this therapeutic modality in patients with dissemin-

[1] Received Nov 16, 1973; revised Feb 14, 1974; accepted Feb 25, 1974.

[2] Compounds used in this study were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

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[6] The authors wish to thank Dr. Arthur Serpick for his assistance in the management of these patients, Dr. Peter Wiernik for reviewing the manuscript, and Ms. Delores Norris for secretarial assistance.

[7] Andrews GS, Sittersun BW, White DA, et al. Summary of clinical total body irradiation program. Research report from the Oak Ridge Institute of Nuclear Studies, Medical Division, 1962.

TABLE 3.—Effects of NSC-143019 on various kinds of ascites tumors in mice*

Tumor†	Dosage (mg/kg/day × 5, ip)	MST (days) (treated/control)	%ILS	Average body weight change (g), Days 1–5 (treated/control)
L1210/6MP/R	25	12.7/9.3	37	1.3/2.0
L1210/ara-C/R	25	10.7/8.2	30	1.7/4.2
	50	11.5/8.2	40	1.3/4.2
L1210/MTX/R	25	9.2/10.2	0	–1.8/–1.6
	50	9.0/10.2	0	–2.8/–1.6
L1210/DM/R	25	8.8/8.6	2	–1.2/0.8
	50	8.6/8.6	0	–1.6/0.8
6C3HED/OG	10	30.0/14.0	114	–1.0/–0.2
	20	24.2/14.0	73	–1.4/–0.2
6C3HED/RG	25	8.8/11.0	0	–1.8/2.4
MH134	25	11.2/15.8	0	–2.8/1.2
MH129p	25	26.4/22.6	17	–0.4/0.6
	50	19.7/22.6	0	0.4/0.6

*One million L1210 cells or 1×10^7 cells of other kinds of tumors were transplanted ip, and drugs were administered ip daily for 5 days starting 24 hours after tumor transplantation. Five mice per group.

†For explanation of abbreviations *see* Materials and Methods.

TABLE 4.—Effects of NSC-143019 on the growth of sc transplanted tumors in mice*

Tumor	Dosage† (mg/kg/day)	Route	Mean tumor size: Day of assessment	Mean tumor size: mm (treated/control)	Inhibition (%)	Average body weight change (g), Days 1–5 (treated/control)
S180	25 × 5	ip	16	10.5/19.3	46	–0.8/3.2
	50 × 5	ip	16	10.0/19.3	48	–2.7/3.2
B16	10 × 5	ip	15	17.5/21.5	19	–0.2/0.1
	25 × 5	ip	15	17.0/21.5	21	–0.6/0.1
	50 × 5	ip	15	11.2/21.5	48	–0.8/0.1
6C3HED/OG	25 × 10	ip	11	10.7/17.2	38	2.3/2.6
	50 × 10	ip	11	8.1/17.2	53	–0.3/2.6
MH134	25 × 10	ip	12	15.8/17.6	10	1.2/1.3
	50 × 10	ip	12	13.1/17.6	26	0.8/1.3
MH129p	25 × 10	ip	12	14.9/16.1	7	0.8/1.1
	50 × 10	ip	12	13.2/16.1	18	0.2/1.1
L1210	50 × 5	oral	7	7.1/10.5	32	1.8/2.2
	100 × 5	oral	7	4.6/10.5	56	1.2/2.2
Ehrlich	100 × 5	oral	9	7.3/13.3	45	–2.9/1.0
MH134	100 × 10	oral	12	11.9/17.6	32	1.2/1.3
MH129p	100 × 10	oral	12	12.7/16.1	21	0.4/1.1

*B16 melanoma and S180 were transplanted sc using trocar pieces (2 mm^3 in size), and 1 million L1210 cells and 10 million of other kinds of ascitic tumor cells were transplanted sc. Five mice per group.

†Drug was injected ip or orally daily for 5 or 10 days starting 24 hours after tumor implantation.

Antagonism of antitumor effect by nicotinic acid.—Repeated ip doses of 50 mg/kg of NSC-143019, starting 24 hours after sc implantation of L1210, prolonged the survival time of the mice and reduced the tumor sizes. In such animals, the simultaneous administration of nicotinic acid by mixing in drinking water (1 mg/ml) almost completely prevented the antitumor effects of NSC-143019 (fig 4). Similar antagonism between NSC-143019 and nicotinic acid was demonstrated in the mice inoculated sc with C1498 leukemic cells.

Antagonism in tissue culture.—The growth of HeLa S3 cells was significantly depressed (dose-dependently) at concentrations of 1.0 and 100 μg/ml of NSC-143019 or NSC-4728 (fig 5). The simultaneous addition of equimolar amounts of nicotinic acid to the culture medium almost completely reversed the growth-inhibitory activity of the two drugs.

Cytotoxic Activity

The ip doses of NSC-143019 produced a decrease in the number of mitotic cells until about 15 hours after the administration when compared to those of the control animals, and thereafter the number of mitotic cells increased in the treated animals (data not shown). Destructive figures of tumor cells appeared less prominently from 18 to 24 hours after administration of the single dose of 50 or 100 mg/kg of NSC-143019. The tumor cells undergoing destructive changes were characterized by the enlargement of the cell body with many cytoplasmic granules and vacuoles. These features were somewhat different from those which appeared in the tumor cells treated with mitomycin C and cytosine arabinoside (20). The striking mitotic or chromosomal abnormalities, which were typically observed in the tumor cells treated with cytosine arabinoside or mitomycin C, were rarely found in the tumor cells of the mice treated with NSC-143019.

Immunosuppressive Effects

Effect of hemagglutinin production.—As shown in figure 6, ip treatment with NSC-143019 or NSC-4728 daily for 4 days starting 24 hours after the antigen injection resulted in a significant decrease in antibody titers occurring 5 days after immunization of BDF_1 mice with SRBC. NSC-143019 at a dose of 50 mg/kg and NSC-4728 at a dose of 300 mg/kg completely suppressed the antibody production, and the degree of inhibition was reduced by decreasing the amount of the compounds administered (fig 6). A more marked depression of the production of hemagglutinin was obtained when NSC-143019 or NSC-4728 was given 2 days after the antigen. Further increase in the interval between the antigen and the compound administration resulted in the proportional decrease in depressive activity. When the serum antibody titers were evaluated 9 days after the antigen was given, both drugs given even 2 days after the antigen were no longer effective. In C3H/He mice immunized with 1×10^8 SRBC, the combined sc administration of nicotinic acid at doses of 1, 10, and 50 mg/kg antagonized (dose-dependently) the suppressive effect of 50 mg/kg of NSC-143019 (fig 7).

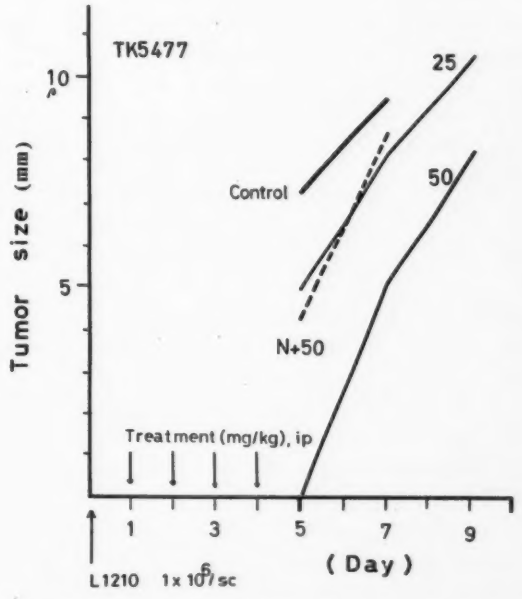

FIGURE 4.—Effect of NSC-143019 (TK5477) on the growth of L1210 leukemia implanted sc and the reversal by nicotinic acid. N + 50 = nicotinic acid mixed in drinking water (1 mg/ml) and administered for 4 days starting on the day 50 mg/kg of NSC-143019 is given.

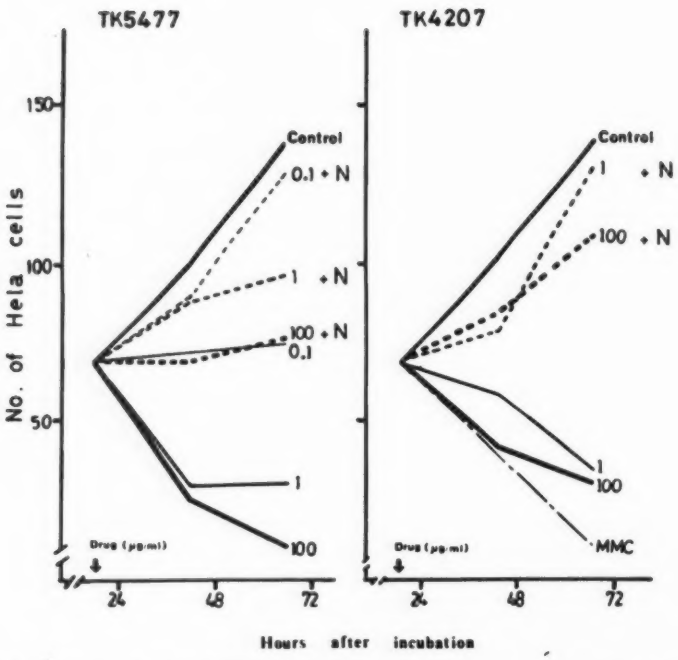

FIGURE 5.—Effects of NSC-143019 (TK5477) and NSC-4728 (TK4207) on the growth of HeLa cells and the reversal by nicotinic acid in vitro. N = nicotinic acid, given in the same quantity as NSC-143019 or NSC-4728. M199 contained 0.25 μg/ml of nicotinic acid and nicotinamide as constituents. The additional administration of nicotinic acid to the medium at a concentration of 100 μg/ml had no influence on the growth of HeLa cells. MMC = mitomycin C, 10 μg/ml. Numbers headed on each line indicate the concentrations of drug (μg/ml) added.

TABLE 5.—Effect of a single injection of NSC-143019 on the primary antibody-forming cell (PFC) responses in spleens of mice immunized with sheep erythrocytes (SRBC)

Antigen*	NSC-143019 (200 mg/kg, ip) given on the indicated day after antigenic stimulation	PFC response on Day 4†	
		PFC/spleen	PFC/10^5 nucleated spleen cells
SRBC	None	620,000 ± 99,454	432,190 ± 82,007
None	None	500 ± 76	396 ± 76
SRBC	1	123,480 ± 33,895	77,788 ± 20,211
SRBC	2	26,000 ± 8071	22,799 ± 7313
SRBC	3	156,520 ± 24,207	139,735 ± 19,221

*C3H/He female mice received iv injections of 1×10^8 SRBC.

†No. of PFCs was assessed 4 days after immunization. Averages of 5 mice ± standard deviation.

TABLE 6.—Effects of NSC-143019 and 6-MP on the GVH reaction in adult F_1 mice*

Transfer of spleen cells	Treatment† (mg/kg/day × 5)	Spleen index (mg/g body weight) (mean of 5 mice ± SE)	Inhibition‡ (%)
No	None	3.36 ± 0.26	—
Yes	None	9.59 ± 0.59	—
Yes	NSC-143019, 10	5.27 ± 0.17	69
Yes	NSC-143019, 20	6.07 ± 0.50	57
Yes	NSC-143019, 40	6.68 ± 0.39	47
Yes	NSC-143019, 40 + nicotinic acid, 10§	6.83 ± 0.92	44
Yes	6-MP, 20	5.17 ± 0.14	71
Yes	6-MP, 40	3.99 ± 0.30	90

*(C57BL/6 × DBA/2)F_1 mice received iv injections of C57BL/6 spleen cells (4×10^7) and their spleens were sampled 10 days later.

†Drugs were injected ip for 5 consecutive days starting 48 hours after cell transfer.

‡Inhibition $= \left[1 - \frac{\text{spleen index of treated mice—spleen index of normal mice}}{\text{spleen index of untreated mice—spleen index of normal mice}}\right] \times 100.$

§Nicotinic acid given iv.

Effect on PFC response.—Groups of female C3H/He mice were immunized iv with 1×10^8 SRBC and treated ip with a single dose of 200 mg/kg of NSC-143019 on the first, second, or third day after the antigen. The PFC responses were measured 4 days after immunization. The most marked depression of the PFC responses was observed in the mice treated with NSC-143019 2 days after antigenic stimulus (table 5).

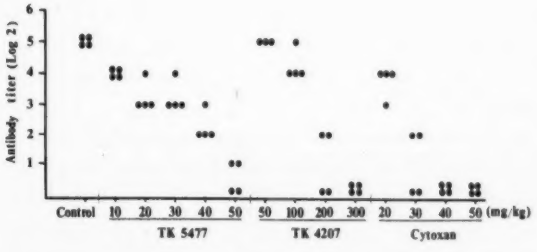

FIGURE 6.—Effects of NSC-143019 (TK5477), NSC-4728 (TK4207), and cyclophosphamide (Cytoxan) on the antibody production in BDF_1 female mice. BDF_1 mice were immunized iv with 1×10^8 SRBC. Drugs were injected ip for 4 consecutive days starting 24 hours after immunization. Hemagglutinin titer of each mouse immunized with SRBC was measured 5 days after the antigen. Each point indicates 1 mouse.

Effect on GVH reaction.—Forty million spleen cells taken from donor C57BL/6 mice were injected iv into groups of BDF_1 mice, resulting in a significant increase in the weight of the spleen 10 days later. The daily ip doses of 10, 20, and 40 mg/kg of NSC-143019 for

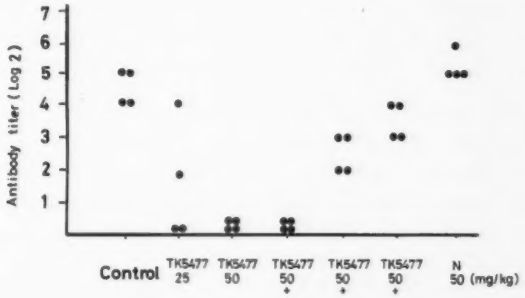

FIGURE 7.—Effect of the simultaneous administration of nicotinic acid on the immunosuppressive activity of NSC-143019 (TK5477) in C3H/He female mice. Mice were immunized iv with 1×10^8 SRBC. NSC-143019 was injected ip and nicotinic acid (N) was injected sc at the time of NSC-143019 treatment. Hemagglutinin titer of each mouse (each point) immunized with SRBC was measured 5 days after the antigen.

5 days, starting 48 hours after the spleen cell transfer, uniformly depressed the increase in spleen index. The same procedure with 20 and 40 mg/kg of 6-MP depressed the spleen index somewhat more markedly. The combination of 10 mg/kg of nicotinic acid given daily through an iv route along with NSC-143019 did not significantly affect the depression of the spleen index by NSC-143019 (table 6).

DISCUSSION

NSC-143019 exhibited considerable antitumor activity in mice (tables 3 and 4). The minimum effective dose of the drug given by the ip route, for increasing the survival time of mice bearing ascitic L1210, was approximately 20 mg/kg, whereas that of NSC-4728 was about 100 mg/kg. The relative effectiveness of NSC-143019 against L1210 was then approximately five times greater than that of NSC-4728. It was interesting that NSC-143019 was markedly inhibitory against L1210 leukemias resistant to 6-MP and cytosine arabinoside but was not inhibitory against those resistant to methotrexate and daunorubicin or against lymphosarcoma resistant to L-asparaginase (6C3HED/RG). Cytologic studies have shown that NSC-143019 affects mainly the tumor cells in the logarithmic phase of proliferation without showing prominent cytopathic effects on the tumor cells, and the recovery of growth in the tumor cells is rather rapid if no increased administration of NSC-143019 is given. These facts imply that the drug is likely to have an antimetabolic action on the proliferating tumor cells.

NSC-143019 was relatively less toxic to normal mice (table 2, fig 1). The repeated administrations of the drug at 150 mg/kg for 10 days did not result in any acute deaths of C3H/He male mice. However, the same dose was intolerable to the mice bearing X5563 myeloma. This suggests a difference in the metabolic patterns of NSC-143019 between normal and tumor-bearing animals. The toxic manifestations that appeared in mice after treatment with large doses of the drug were in close similarity to those of NSC-4728 as reported by Humphrey et al (4). In addition, autopsy findings have indicated that repeated administrations of NSC-143019 at daily ip doses of 1 mg/kg for 3 months result in marked lipophilia in the bone marrow, appearance of normoblasts in the spleen, and atrophy of the thymus in rats.[5] However, the subacute toxicity of NSC-143019 was reported to be weaker in mice than in rats.[5] Such a difference observed between mice and rats in sensitivity to NSC-143019 may be caused by a rapid rate of accumulation of toxic metabolites in rats resulting abruptly in the death of the animals.

Immunosuppression of thiadiazole derivatives has not been reported yet. NSC-143019 exhibited a marked effect in suppressing serum antibody production in mice against SRBC. NSC-4728 was also immunosuppressive to a similar extent. Furthermore, NSC-143019 interfered with the GVH response elicited in adult (C57BL/6 × DBA/2)F_1 mice by administering the parental C57BL/6 spleen cells (table 6). From these findings, it was reasonable to consider that NSC-143019 exerted its effects especially on the function of tissues associated with immune responses. This speculation is supported by histopathologic findings indicating destruction of hematopoietic and thymus tissues in the mice that died from large doses of NSC-143019.[5] The greatest suppression of the serum hemagglutinin and PFC responses in mice occurred when NSC-143019 was administered 2 days after antigenic stimulation. These facts imply that NSC-143019 may damage the immunocompetent cells as well as the tumor cells at the logarithmic phase of proliferation. It is therefore probable that the suppressed immune response is attributed to the decreased number of potent immunocompetent cells, and that antitumor and immunosuppressive activities of NSC-143019 are working with a similar mode of action.

The antitumor and immunosuppressive activities of NSC-143019, as well as the toxicity to the hosts, could be completely abolished by the simultaneous administration of an equimolar amount of nicotinic acid. The mode of action of thiadiazole derivatives has been investigated and discussed with relevance to this nicotinamide antagonism (4–6,13,24,25). Teratogenic activity of NSC-143019 as well as NSC-4728

[5] Miyaji T. Personal communication.

was also protected by nicotinamide.[9] The extent of protection by nicotinic acid of the antitumor and immunosuppressive activities of NSC-143019 appeared to be of the same order as that of NSC-4728. This finding suggests that the injurious action of NSC-143019 to tumor cells or lymphoid cells may be caused by the same or similar metabolic phenomena as in NSC-4728.

LIST OF COMPOUNDS

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6; daunomycin (NSC-82151)

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-; MTX; amethopterin

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

Nicotinic acid: NSC-169454; CAS reg. No. 59-67-6; 3-pyridinecarboxylic acid; Niacin

NSC-4728: CAS reg. No. 4005-51-0; 1,3,4-thiadiazole, 2-amino-; TK-4207

NSC-143019: CAS reg. No. 26907-37-9; 1,3,4-thiadiazole, 2,2'-(methylenediimino)bis-; TK-5477

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Antitumor Activity and Mode of Action of 2-Thio-6-azauridine (NSC-146268) on L1210 Leukemia [1,2,3]

L. H. Li, G. L. Neil, T. E. Moxley, and E. J. Olin [4,5,6]

SUMMARY

This study attempts to evaluate the antitumor effect of 2-thio-6-azauridine against L1210 leukemia and to elucidate its mechanism of action. This drug has a cytotoxic effect on L1210 leukemic cells growing in culture with 50% and 90% inhibition dose values of 0.06–0.11 and 0.23–0.46 μg/ml, respectively. It is also active against L1210 leukemia in vivo. At 0.08 mM/kg/day for 9 days, approximately a 30% increase in lifespan was observed. The cytotoxic effects of this drug on L1210 leukemic cells both in vitro and in vivo approach those achieved with 6-azauridine, which was used as a reference compound in this study. Over a 4-hour period of incubation, 2-thio-6-azauridine significantly inhibited orotic acid transformation and subsequent incorporation but did not inhibit the incorporation of thymidine, uridine, or leucine into macromolecules. This inhibition was almost completely prevented by the addition of uridine, and was partially prevented by cytidine. Neither deoxyuridine, deoxycytidine, adenosine, nor guanosine had any effect. 2-Thio-6-azauridine was found to inhibit the transformation of orotic acid to phosphorylated products of uridine by inhibiting orotidylic acid decarboxylase. Thus, all evidence showed that 2-thio-6-azauridine, after conversion to the 5′-phosphate, interrupted the de novo synthesis of pyrimidine nucleotides by specific inhibition of the enzyme orotidylic acid decarboxylase.

[Cancer Chemother Rep 58:345–352, 1974]

2-Thio-6-azauridine and 2-thio-6-azauracil [7] were chemically synthesized by Dr. J. H. Hunter in the Upjohn research laboratories. We are reporting the antitumor effect and a proposed mechanism of action of 2-thio-6-azauridine, both in vitro and in vivo, on the experimental L1210 tumor system. 6-Azauridine [8] was used in these studies for the purpose of comparison.

MATERIALS AND METHODS

Chemicals and medium.—Radioactive thymi-

[1] Received Sept 21, 1973; revised Jan 15, 1974; accepted Jan 24, 1974.

[2] Supported in part by contract PH43-68-1023 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] 2-Thio-6-azauridine: CAS reg. No. 27089-56-1; *as*-triazine-3,5(2*H*, 4*H*)-dione, 2-β-D-ribofuranosyl 3-thio-.

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[6] The authors wish to thank Dr. J. H. Hunter and Mr. H. Skulnick for supplying the compounds 2-thio-6-azauridine and 2-thio-6-azauracil; Mrs. J. W. Culp for her skillful technical assistance; and Ms. E. L. Clark and Ms. M. J. Campbell for their help in the preparation of this manuscript.

[7] 2-Thio-6-azauracil: NSC-146269; CAS reg. No. 626-08-4; *as*-triazine-3,5(2*H*,4*H*)-dione, 3-thio-.

[8] 6-Azauridine: NSC-32074; *as*-triazine-3,5(2*H*,4*H*)-dione, 2-β-D-ribofuranosyl-.

TABLE 1.—Effect of 2-thio-6-azauridine on the macromolecular synthesis of L1210 cells in culture*

2-Thio-6-azauridine concentration (μg/ml)	Thymidine-^{3}H		Uridine-^{3}H		Orotic acid-^{3}H		Leucine-1-^{14}C	
	SA†	%‡	SA	%	SA	%	SA	%
0	875	100.0	550	100.0	312	100.0	16.1	100.0
2	747	85.4	557	101.3	118	37.8	15.5	96.3
10	753	86.1	522	94.9	44	14.1	13.2	82.0
50	914	104.5	519	94.4	28	9.0	22.0	136.6

*L1210 cells (about 5×10^5 cells/ml) were incubated at 37 C simultaneously with 2-thio-6-azauridine at different concentrations and a given labeled metabolite (thymidine-^{3}H or uridine-^{3}H [25 millicuries/12 mμM/ml of cells]; leucine-1-^{14}C [0.5 millicuries/200 mμM/ml]; and orotic acid-^{3}H [2.5 millicuries/18 mμM/ml]) for up to 4 hrs (within the initial rate of incorporation and the standard deviation of 5%). The DNA and RNA fractions were obtained by extracting the washed cell pellet twice with 1 ml of 0.5N perchloric acid (PCA) for 20 min at 70 C and the protein fraction was obtained by digesting the washed cell pellet with 2 ml of 1N NaOH at 37 C overnight. The extracts were then used for counting radioactivities and determining the nucleic acid and protein contents.

†SA = specific activity (cpm/μg of DNA, RNA, or protein) for a period of 2-hr incorporation.

‡% = Percent of control calculated from the specific activity of incorporation.

dine, uridine, orotic acid, orotidine-5′-phosphate, and leucine were obtained from New England Nuclear Corporation (Boston, Mass). Tritiated 6-azauracil[9] was purchased from Schwartz/Mann (Orangeburg, NY). RPMI 1634 medium and supplements were supplied by Grand Island Biological Company (Grand Island, NY).

L1210 cell-growth study.—L1210 cells were maintained in basal medium developed by Moore et al (1); the details of the procedure were described in the legends. To determine ID50 and ID90 values, 5 ml of L1210 cells (5×10^3 cells/ml) was incubated with the drug at 37 C for 3 days, and cell growth was determined (2).

Antitumor activity.—The methods used in studying the effects of agents on the survival of L1210 leukemic mice were similar to those described by Drug Research and Development (formerly the Cancer Chemotherapy National Service Center), DCT, NCI (3). A 25% increase in lifespan (ILS) is considered to be significant antitumor activity.

Macromolecular synthesis.—The details of the procedure are described in table 1. RNA content was determined by the orcinol method (4); DNA content was determined by Burton's modification of the diphenylamine method (5); protein content was determined according to the procedure of Lowry et al (6).

Enzyme assays.—A crude cell-free extract of either erythrocyte-removed ascitic cells or L1210 cells in culture was used as the source of enzymes. The isolation procedure was described elsewhere (7). Uridine kinase was determined according to the procedure of Chu and Fischer (8). The reaction was terminated by heating for 2 minutes in a boiling water bath, and the phosphorylated derivatives were separated by thin-layer chromatography (silica gel) via the solvent system of methyl ethyl ketone:acetone:water in a 7:2:1 ratio. Orotidine-5-phosphate (OMP) decarboxylase activity was determined according to the procedure of Holmes (9). The reaction mixture was, however, modified as described in table 2, and the reaction was carried out at 37 C instead of 25 C.

RESULTS

Antitumor activity and cytotoxicity.—The cytotoxicity of four drugs against L1210 cells in culture is shown in table 3. 2-Thio-6-azauridine is somewhat less cytotoxic than 6-azauridine. 6-Azauracil and 2-thio-6-azauracil, the bases, are much less active. The effect of 2-thio-6-azauridine on the survival of L1210 leukemic mice is summarized in table 4. Daily intraperitoneal (ip) doses of 2-thio-6-azauri-

[9] 6-Azauracil: NSC-3425; CAS reg. No. 461-89-2; *as*-triazine-3,5(2*H*,4*H*)-dione.

TABLE 2.—Effect of 2-thio-6-azauridine on OMP decarboxylase of L1210 cells in culture and in vivo*

2-Thio-6-azauridine concentration (μg/ml)	L1210 cells in culture		L1210 cells in vivo	
	SA†	Inhibition (%)	SA	Inhibition (%)
0 (control)	12.11 ± 1.05	0	5.51 ± 0.80	0
10	9.76 ± 3.32	19.4	2.55 ± 1.91	53.8
25	5.12 ± 0.48	57.7	1.14 ± 0.07	79.2
50	0.12 ± 0.37	98.9	0.54 ± 0.12	90.2

*The reaction mixture (2.0 ml) contained ATP, 2.25×10^{-3} M; $MgCl_2$, 3.5×10^{-3} M; tris buffer, 100 mM (pH 7.95); protein (the high-speed supernatant [105,000 × *g* for 60 min] of crude cell-free extract isolated from L1210 cells either in vitro or in vivo), 4.0 mg; and 2-thio-6-azauridine, different concentrations (0–50 μg). The reaction system was incubated at 37 C for 60 min with continual gentle shaking. The OMP decarboxylase reaction was started by the addition of 0.1 ml of OMP-carboxyl-^{14}C (0.2 mM, 3.5×10^4 cpm) to the reaction mixture and incubation at 37 C. At the appropriate time (10, 20, and 30 min), 0.5-ml amounts of the samples were pipetted into tubes containing 10% trichloroacetic acid (TCA) and were heated in a boiling water bath for 5 min to expel the last traces of $^{14}CO_2$. After cooling and centrifugation, 0.1 ml of the sample was used for the radioactivity determination.

†SA = specific activity (units of enzyme/mg of protein). A unit of OMP decarboxylase activity has been defined by Lieberman et al (10) as the amount of enzyme required to decarboxylate 1 mμM of OMP-7-^{14}C/hr calculated from the negative slope of initial reaction rate (10–30 min) and corrected via linear regression.

TABLE 3.—Cytotoxicity against L1210 cells in culture*

Drugs	Cytotoxicity (μg/ml)	
	ID50	ID90
2-Thio-6-azauridine	0.06–0.11	0.23–0.46
2-Thio-6-azauracil	26.0	48.0
6-Azauridine	0.05	0.11
6-Azauracil	> 50.0	—

*Five-ml aliquots of cells (about 5×10^3 cells/ml) were incubated in sterilized screw-cap culture tubes with and without drugs at 37 C for 3 days. Cell growth was determined on a model-A Coulter counter and % inhibition was calculated from comparison with controls (no drug).

dine (0.8 mM/kg) for 9 days yielded a 29% ILS. A direct comparison of the antitumor effects of 2-thio-6-azauridine and 6-azauridine was made. At equimolar doses, 2-thio-6-azauridine is somewhat less effective and less toxic (in terms of weight loss) than 6-azauridine (table 4). The lack of an observed dose response with 2-thio-6-azauridine may reflect its relative insolubility.

Effect of 2-thio-6-azauridine on macromolecular synthesis of L1210 leukemia.—As shown in table 1, no significant effect was observed on the incorporation of thymidine-^3H into DNA, uridine-^3H into RNA, or leucine-1-^{14}C into protein when the cells were incubated with 2-thio-6-azauridine (at up to 50 μg/ml for up to 4 hours). However, when the cells were incubated with 2-thio-6-azauridine at a level of 2 μg/ml, orotic acid incorporation was inhibited over 60%, and inhibition appeared to be dose-related. 2-Thio-6-azauracil showed little inhibitory effect. These results almost parallel those of cell-growth inhibition described in table 3. In leukemic BDF_1 mice, similar results were obtained when 2-thio-6-azauridine was administered ip at a dose of 200 mg/kg. Only orotic acid incorporation was significantly inhibited (from 70% to 90%) in all of the tissue studied (data not presented).

Effect of various metabolites on 2-thio-6-azauridine-inhibition of orotic acid incorpora-

TABLE 4.—Antitumor activity of 2-thio-6-azauridine and 6-azauridine against L1210 leukemia in vivo*

Dose† (mM/kg/day)	2-Thio-6-azauridine			6-Azauridine		
	Mean survival ±SD‡ (days)	%ILS§	Average weight change (g/mouse)¶	Mean survival ±SD (days)	%ILS	Average weight change (g/mouse)
0	8.0 ± 0.5	—	+1.2	8.0 ± 0.5	—	+1.2
0.8	10.3 ± 1.6	29	0	10.4 ± 1.0	30	−0.3
1.2	10.1 ± 0.7	26	−0.1	11.2 ± 1.9	40	−0.5
1.6	9.8 ± 0.8	23	−0.2	11.9 ± 0.9	49	−1.5

*Mice were inoculated ip with 1×10^5 ascitic L1210 cells/mouse on Day 0.
†Treatment (ip) was initiated on Day 1 and continued daily for 9 days.
‡SD = standard deviation.
§% ILS was calculated from mean survival rates of control and treated groups (eight mice per group).
¶Animals were weighed on Day 0 and again on Day 5.

TABLE 5.—Effect of various metabolites on the inhibition of orotic acid incorporation by 2-thio-6-azauridine in L1210 leukemia in vitro and in vivo

System	Organ	Metabolite	Specific activity (cpm/μg of RNA)		
			Control	2-Thio-6-azauridine	2-Thio-6-azauridine/controls (%)
L1210 cells in culture*		None	9820	3570	36.4
		Uridine	8220	7800	95.0
		Cytidine	8580	5320	62.0
		Deoxyuridine	9700	3200	33.0
		Deoxycytidine	9700	3220	33.2
L1210 leukemic mice†	Ascitic cells	None	1476	152	10.3
		Uridine	590	612	104.0
	Liver	None	459	117	25.5
		Uridine	464	216	46.6
	Spleen	None	275	84	30.5
		Uridine	167	201	120.0

*For the in vitro study, L1210 cells (about 5×10^5 cells/ml) were incubated at 37 C simultaneously with 2-thio-6-azauridine (1 μg/ml of cells), orotic acid-^{3}H (1.5 millicuries/18 mM/ml), and a given metabolite (5 μg/ml of cells) for up to 4 hrs. The rest of the procedure is described in table 1.

†For the in vivo study, L1210 leukemic mice (bearing about 1×10^5 cells/animal) were injected ip with 0.5 ml of samples composed of 4 mg of 2-thio-6-azauridine (or 0.9% NaCl), 20 mg of uridine (or 0.9% NaCl), and orotic acid-^{3}H (10 millicuries/2 mg). Animals (three mice per group) were sacrificed 1 hr later, and ascites, liver, and spleen were collected. The rest of the procedure is described in table 1. The doses of 2-thio-6-azauridine and uridine used in this study were 200 mg/kg and 1 g/kg, respectively.

tion into L1210 leukemia in vitro and in vivo, and of L1210 cell growth in culture.—The inhibition by 2-thio-6-azauridine of orotic acid incorporation into L1210 cells growing in culture is greatly reduced by the simultaneous addition of uridine (uridine:2-thio-6-azauridine = 5:1 wt/wt) and, to a lesser extent, by the addition of cytidine. Neither adenosine, guanosine (LH Li, unpublished data), deoxyuridine, nor deoxycytidine had any significant effect, as summarized in table 5. A similar phenomenon was observed in L1210 leukemic mice (table 5). When animals simultaneously received a dose of uridine (1 g/kg), the inhibition of orotic acid-^{3}H incorporation by 2-thio-6-azauridine (200 mg/kg) was completely prevented in ascitic cells and the spleen but was only partially reduced in the liver. Similar results, as seen in figure 1, were also obtained in the study of L1210 cell growth in culture. In the presence of uridine (5 μg/ml), the inhibition of cell growth by either 2-thio-6-azauridine or 6-azauridine (1 μg/ml) is almost completely eliminated.

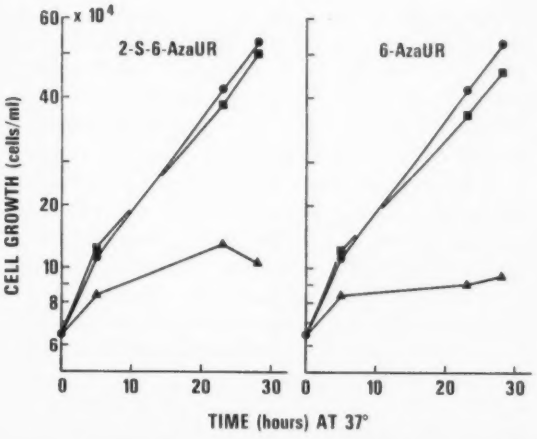

FIGURE 1.—Effect of uridine on the inhibition of L1210 cell growth in culture by 2-thio-6-azauridine and 6-azauridine. Four-ml aliquots of cells (about 6 × 10⁴ cells/ml) were pipetted into sterilized plastic culture tubes fitted with screw caps. The experiment was initiated by the simultaneous addition of 0.5 ml of medium or 2-thio-6-azauridine or 6-azauridine (10 μg/ml) and 0.5 ml of medium or uridine (50 μg/ml) and incubation at 37 C. Cell growth was determined (average of triplicate samples) by means of electronic particle counting. ●—● = Controls; ▲—▲ = 2-thio-6-azauridine or 6-azauridine (1 μg/ml); ■—■ = drug + uridine (5 μg/ml).

Effect of 2-thio-6-azauridine on the transformation of orotic acid in L1210 leukemia in vitro and in vivo.—Cells were exposed to the drug (5 μg/ml) for 1–4 hours (37 C) and acid-soluble fractions were isolated and separated by paper chromatography with a solvent system composed of 95% ethanol:1 M ammonium acetate (5:2), pH 7.5. The results in figure 2*A* indicate that although 2-thio-6-azauridine does not affect the entrance of orotic acid into the cells, it does significantly inhibit the transformation of orotic acid into phosphorylated products of uridine (apparently uridine-5′-triphosphate is the main product). In leukemic BDF_1 mice, as in the in vitro situation, the formation of phosphorylated uridine from orotic acid is also blocked by this drug, as shown in figure 2*B*.

Effect of 2-thio-6-azauridine on OMP decarboxylase activity and on the uridine kinase of L1210 cells in culture and/or in vivo.—The degree of phosphorylation of 6-azauridine-^{3}H by the crude cell-free extract isolated from L1210 cells growing in culture was determined by 30- and 60-minute periods of incubation at 37 C. Based on these data, a preincubation time of 60 minutes was chosen for determining the effect of 2-thio-6-azauridine at different concentrations on OMP decarboxylase under the assay conditions described in table 2. The reaction was started by the addition of OMP-carboxyl-^{14}C and was stopped after 30 minutes' incubation (within the initial rate of reaction). The results, as shown in table 2, indicate that 2-thio-6-azauridine, or its metabolic products or both, indeed inhibited the OMP decarboxylase activity of cell-free extracts which were isolated from L1210 cells growing either in culture or in leukemic BDF_1 mice. At the 25-μg/ml level, about 58% and 79% of enzyme activity was inhibited, respectively, and no apparent inhibition was observed without preincubation. Furthermore, no significant effect on the uridine kinase of L1210 cells in culture was observed in the presence of up to 200 μg/ml of either 2-thio-6-azauridine or 6-azauridine (LH Li, unpublished data).

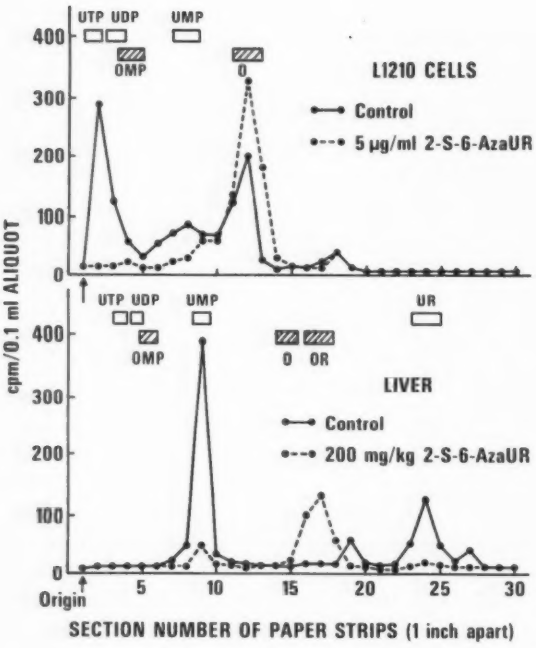

FIGURE 2.—Effect of 2-thio-6-azauridine on the transformation of orotic acid in L1210 leukemia in vitro and in vivo. For in vitro study, cells were exposed to the drug (5 μg/ml) and orotic acid-^{3}H (1.5 millicuries/18 mM/ml) for 1–4 hrs (37 C), and acid-soluble fractions obtained by extracting the cell pellet with 0.2N PCA (4 C) were separated by paper chromatography with a solvent system composed of 95% ethanol:1 M ammonium acetate (5:2), pH 7.5. For in vivo study, leukemic mice (bearing about 1×10^6 cells/animal) were injected ip with 0.5 ml of samples composed of 4 mg of 2-thio-6-azauridine (0.9% NaCl) and orotic acid-^{3}H (10 millicuries/2 μg). Animals (three mice per group) were sacrificed 1 hr later, and livers were collected. Acid-soluble fractions were obtained by extracting the tissue pellet with 0.2N PCA (4 C). To the acid extract containing the free nucleotides 1 mM of UMP was added as marker. The concentration of PCA was adjusted to 1N and the solution heated at 100 C for 20 min. Under these conditions all free uridine nucleotides should be quantitatively converted to the monophosphate (13). The hydrolysates were neutralized with KOH and PCA was removed as potassium perchlorate. Supernatants were lyophilized and redissolved in 0.5 ml of water just prior to paper chromatography with the solvent system 95% ethanol:1 M ammonium acetate (5:2), pH 7.5.

DISCUSSION

2-Thio-6-azauridine was found to be active against L1210 leukemia in vitro and in vivo. On a molar basis, it is about 100 times more active against L1210 cells growing in culture than its corresponding base, 2-thio-6-azauracil (table 3). In an attempt to understand the nature of its activity, the effect of 2-thio-6-azauridine on macromolecular synthesis was studied. For up to 4 hours' incubation, no significant effects on thymidine-^{3}H, uridine-^{3}H, and leucine-1-^{14}C incorporation into their respective macromolecules were observed, but orotic acid incorporation was significantly inhibited even at a level of 2 μg/ml (table 1). This phenomenon was also seen in vivo. These results collectively suggest that 2-thio-6-azauridine inhibits tumor cell growth by interfering with de novo synthesis of pyrimidine nucleotides. The inhibition of orotic acid incorporation into L1210 cells growing in culture (table 5) was almost completely prevented by the simultaneous addition of uridine (uridine:2-thio-6-azauridine = 5:1, wt/wt) and partially prevented by cytidine. Neither adenosine, guanosine, deoxyuridine, nor deoxycytidine had any effect. Similar results were obtained in studies of L1210 cell growth in culture (fig 1) and of orotic acid incorporation into the spleen and ascitic L1210 cells of leukemic BDF_1 mice (table 5). The inhibition of orotic acid incorporation into the liver, however, was only partially prevented by uridine, a phenomenon which remains to be explained. These results suggest that the phosphorylated product rather than the parent compound, 2-thio-6-azauridine, is responsible for its cytotoxic effect on tumor cells. Phosphorylation of 2-thio-6-azauridine probably occurs via the uridine kinase system (11) since only uridine and, to a lesser extent, cytidine prevented 2-thio-6-azauridine inhibition of both orotic acid incorporation (table 2) and cell growth (fig 1). The affinity of uridine (or cytidine) for uridine kinase may be much stronger than that of 2-thio-6-azauridine, hence no significant effect of this drug on the phosphorylation and incorporation of uridine (table 1) would be observed. Whether this drug is phosphorylated only to a monophosphate, as in the case of 6-azauridine (12), or is phosphorylated further to diphosphate and triphosphate derivatives and is ultimately incorporated into polynucleotides remains to be determined when a radioac-

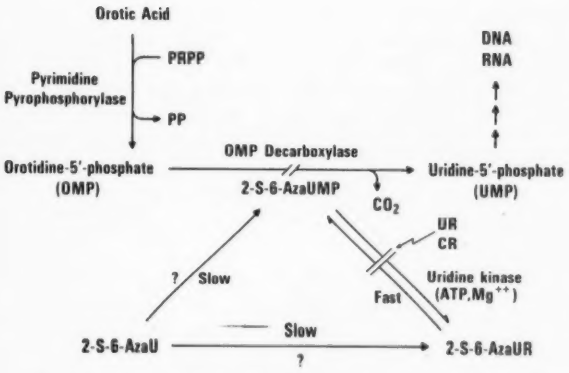

FIGURE 3.—A possible mechanism of action of 2-thio-6-azauridine on L1210 leukemia.

tive drug is available. Incorporation seems unlikely since the inhibition of L1210 cell growth in culture can readily be reversed by either the addition of uridine or by the removal of the drug after a period of 2 hours of contact with cells (LH Li, unpublished data).

2-Thio-6-azauridine was found to significantly inhibit the in vitro and in vivo transformation of orotic acid into phosphorylated products of uridine (fig 2). This again suggests that the active metabolite of this drug is its 5′-phosphate ester. Further evidence suggesting that 2-thio-6-azauridine-5′-phosphate is the actual inhibitor was obtained by studying the decarboxylation of orotidylic acid in an unfortified reaction mixture. By combining the reaction mixture for assaying nucleotide kinases (8), after a 60-minute preincubation period to allow the phosphorylation of 2-thio-6-azauridine to take place, with the OMP decarboxylase system, a marked inhibition of this enzyme is observed in cells obtained from culture and leukemic mice (table 2). 2-Thio-6-azauracil, the base, has considerably less cytotoxic activity (table 3). Apparently, conversion of 2-thio-6-azauracil to 2-thio-6-azauridine-5′-phosphate (via the pathways normally followed by uracil to its ribonucleotide) is a rather slow process. Thus, all evidence (summarized in fig 3) suggests that after conversion to the 5′-phosphate, 2-thio-6-azauridine, as does 6-azauridine (12,14), interrupts the de novo synthesis of pyrimidine nucleotides by specific inhibition of the enzyme OMP decarboxylase.

So far, our results indicate that the mechanism of action of 2-thio-6-azauridine against L1210 leukemia is more or less similar to that of 6-azauridine, a clinically useful drug in the treatment of psoriasis, mycosis fungoides, and certain types of tumors such as chronic myelogenous leukemias and acute leukemias (15,16). However, 2-thio-6-azauridine is somewhat less active against L1210 leukemia both in culture and in vivo than 6-azauridine (tables 3 and 4). Whether this drug possesses any other desirable characteristics remains to be determined.

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Estradiol Mustard (NSC-112259) and Phenester (NSC-116785): Possible Mediation of Action by Estrogen Binding Protein [1,2,3]

Richard B. Everson,[4] Roger W. Turnell,[5] James L. Wittliff, [5] and Thomas C. Hall [6,7]

SUMMARY

The potential of estrogen binding proteins in the cytoplasm to mediate the action of the steroidal alkylating agent, estradiol mustard, was investigated using competition studies of this drug for specific binding sites on the receptor. Estradiol mustard inhibited the specific binding of ^{3}H-estradiol-17β to uterine cytosol receptors while the alkylating moiety, phenester, was without effect. Inhibition of binding by estradiol mustard was observed at greater than 10^{-6} M and was complete at 10^{-3} M. From these data an I_{50} of 3×10^{-5} M was determined and a K_d of 3×10^{-6} M was calculated for estradiol mustard-receptor complex. Sucrose gradient analysis indicated that estradiol mustard blocked the formation of the specific 8S ^{3}H-estradiol-17β-receptor complex of the cytoplasm. The displacement of binding of ^{3}H-estradiol-17β by estradiol mustard observed both on sucrose gradients and in the dextran-coated charcoal assay is not due to liberation of estradiol resulting from breakdown of estradiol mustard.

[Cancer Chemother Rep 58:353–357, 1974]

Estradiol mustard (fig 1), a steroidal alkylating agent comprised of an estradiol-17β nucleus with two cytotoxic chlorphenacyl side groups, was developed by Wall et al (1) from the concept of Larionov et al (2). Recently reported results from our laboratory indicate that estradiol mustard is moderately uterotrophic and causes regression of the R3230AC mammary adenocarcinoma of the rat (3). Vollmer and co-workers have suggested that the steroidal portion might act as a carrier of the cytotoxic moiety, facilitating its passage across cell membranes perhaps even to specific sites of hormonal action (4), by utilizing the hormone receptors in the target tissue (5). This hypothesis, although proposed, has not been examined experimentally. By analogy to estradiol-17β, mediation of the action of estradiol mustard by estrogen-binding protein would involve initially binding to the cytoplasmic estrogen receptor.

[1] Received Jan 24, 1974; revised Apr 18, 1974; accepted Apr 18, 1974.

[2] Supported in part by institutional grant IN-18L from the American Cancer Society, and Public Health Service grant CA-11198 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Estradiol mustard: CAS reg. No. 22966-79-6; estra-1,3,5(10)-triene-3,17β-diol, bis[[*p*-[bis(2-chloroethyl)amino]phenyl]acetate] (lot No. 9306-CYP-50B, Upjohn Co.). Estradiol mustard was reported to contain no free estradiol.

Phenester: CAS reg. No. 31386-35-3; acetic acid, [*p*-[bis(2-chloroethyl)amino]phenyl]-, ethyl ester (lot No. 9306-CYP-32A, Upjohn Co.).

Both drugs were obtained courtesy of Dr. D. Jane Taylor, Breast Cancer Program Coordinating Branch, Division of Cancer Biology and Diagnosis, NCI.

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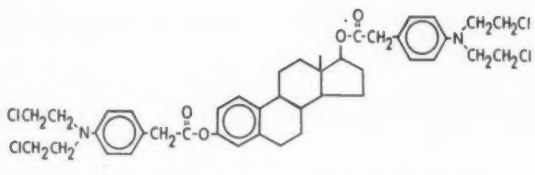

FIGURE 1.—Structure of estradiol mustard.

Accordingly, this study reports determinations of the ability of estradiol mustard to bind to receptors in rat uterine cytosol as measured by the inhibition of binding of the active estrogen, estradiol-17β.

MATERIALS AND METHODS

Estradiol-17β (estra-1,3,5[10]-triene-3, 17β-diol), tritiated either as ^{3}H-[2,4,6,7]-estradiol-17β or ^{3}H-[6,7]-estradiol-17β, with specific activity ranging from 40 to 114 curies/mM, was obtained from the New England Nuclear Corporation and used without further purification. Unlabeled estradiol-17β was obtained from Calbiochem. The nonsteroidal compound CN-55, 945-27 [1-(2-[p-(α-[p-methoxyphenyl]-β-nitrostyryl)phenoxy]ethyl)-pyrolidine, monocitrate] was obtained from Dr. J. R. Reel, courtesy of Parke-Davis Co.

Uteri were removed from 80–130-g Fischer 344 rats, cleaned of connective tissue, blotted, and homogenized in 10 mM tris HCl, 1.0 mM Na_2EDTA, 250 mM sucrose, pH 7.4 or 8.0 buffer, and a 50,000 or 105,000 × g supernatant was prepared as described by Wittliff et al (6). Each cytosol preparation was incubated for 10–30 minutes at 0–3 C with a test compound (unlabeled estradiol-17β, CN-55,945-27, estradiol mustard, or phenester) in the presence of buffer and 20% DMSO or 5% acetone which was used to dissolve the test steroid (total volume, 0.25 ml).[8] This mixture was then transferred to a shell vial containing the ^{3}H-estradiol-17β and incubated for a further 14–20 hours at 0–3 C. Alternatively, in some experiments all of the components were added simultaneously with similar results being obtained. To terminate the incubation and determine the amount of bound ^{3}H-estradiol-17β, a modified charcoal binding technique of Korenman (7), as described by Gardner and Wittliff (8), was used to remove the unbound ^{3}H-ligand, or gradient centrifugation analysis of bound ^{3}H-estradiol-17β was performed using a modified technique of Toft et al (9) as reported by Wittliff et al (6).

[8] Even in the presence of 20% DMSO or 5% acetone in the incubation mixtures estradiol mustard was insoluble at concentrations higher than 1×10^{-3} M.

RESULTS

For this study, we have confirmed the efficacy of the charcoal separation assay reported elsewhere (10) by demonstrating similar time, temperature, and ligand concentration relationships for ^{3}H-estradiol-17β binding to receptors from cytosol (results not presented).

Inhibition of ^{3}H-estradiol-17β binding is essentially complete at a hundredfold excess (2×10^{-7} M) of estradiol-17β (fig 2). Fifty percent inhibition ($[E_t]_{50}$) of binding was achieved by 5–6 × 10^{-9} M unlabeled estradiol-17β. Estradiol

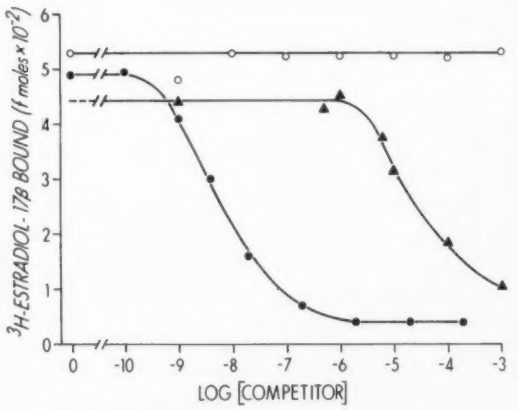

FIGURE 2.—Effect of estradiol mustard and its molecular components on the binding of ^{3}H-estradiol-17β to rat uterine cytosol receptor using the dextran-coated charcoal assay. Uterine cytosols were prepared and preincubated for 30 min at 0 C with the competitor dissolved in 5% acetone at the concentrations indicated in the figure. This mixture was then transferred to a shell vial containing ^{3}H-estradiol-17β (final concentration, 5.6×10^{-9} M) and incubated a further 16 hrs. The bound and free ^{3}H-estradiol-17β were separated as described in Materials and Methods. The protein concentration was 10 mg/ml. Each point is the mean of 3 independent determinations. Competitors: ●——● = unlabeled estradiol-17β, ▲——▲ = estradiol mustard, and ○——○ = phenester.

mustard, at concentrations greater than 10^{-6} M, was effective in inhibiting the binding of 5 nM ^{3}H-estradiol-17β. At 2–3 $\times$ 10^{-5} M estradiol mustard inhibition of ^{3}H-estradiol-17β was ~50% while inhibition of binding was essentially complete at 10^{-3} M (fig 2). Phenester, over a range of 10^{-10} to 10^{-3} M, did not affect the binding of ^{3}H-estradiol-17β to receptor. Using the following relationship devised by Sherman et al [9] it is possible to calculate a dissociation constant for the estradiol mustard-receptor complex.

$$K_c = \frac{K_E[C_t]_{50}}{[E_t]_{50}}$$

where K_c = the dissociation constant for the competitor (in this case, estradiol mustard); K_E = the dissociation constant for the ^{3}H-estradiol-17β, calculated to be 6 $\times$ 10^{-10} for rat uterine cytosol (data not presented); $[E_t]_{50}$ = the concentration of estradiol-17β that inhibits the binding of ^{3}H-estradiol-17β by 50%; and $[C_t]_{50}$ = the concentration of competitor that inhibits the binding of ^{3}H-estradiol-17β by 50%. Using this relationship, we have calculated a dissociation constant of ~3 $\times$ 10^{-6} M for estradiol mustard-receptor complex.

The sedimentation profile seen in figure 3 demonstrates the specificity of binding of ^{3}H-estradiol-17β to 8S cytosol receptors. These results confirm those previously published (11). An estrogen antagonist like CN-55, 945-27 completely inhibits the binding of ^{3}H-estradiol-17β to 8S receptors (fig 3*B*). As shown in figure 3*C*, 20% DMSO had no effect on the binding of ^{3}H-estradiol-17β to the receptor. Similar results were obtained when using DMSO with the charcoal adsorption assay and when binding was carried out with either assay in the presence of 5% acetone (results not presented). Phenester had no effect on the binding of ^{3}H-estradiol-17β (fig 3*D*) confirming the results presented in figure 2. Estradiol mustard at 1 $\times$ 10^{-5} M or 1 $\times$ 10^{-4} M reduced the binding of ^{3}H-estradiol-17β to 8S receptors by 35% and 80% respectively (fig 3 [*E* and *F*]). These reductions compare very well with those seen using the charcoal adsorption assay (compare figs 2 and 3[*E* and *F*]).

A time course of inhibition of ^{3}H-estradiol-17β binding by estradiol mustard was carried out at 4 C, in order to determine whether the inhibition observed was due to free estradiol

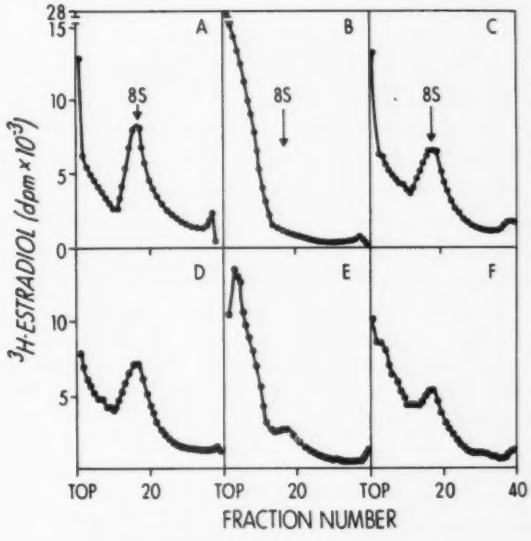

FIGURE 3.—Effect of estradiol mustard, its molecular components, and the antiestrogen CN-55,945-27 on the binding of ^{3}H-estradiol-17β to the 8S receptor of rat uterine cytosol separated by sucrose gradient analysis. Cytosol (0.15 ml) was preincubated for 10 min at 0–3 C with the competitor and then incubated a further 30 min in the presence of 3.0 $\times$ 10^{-9} M ^{3}H-estradiol-17β (total volume, 0.250 ml). The cytosols were then layered on linear 5%–20% sucrose gradients and centrifuged at 308,000 $\times$ g in a Beckman SW 56 Ti rotor for 8 hrs. Each tube was then pierced and 40 6-drop fractions were collected from the bottom of the gradient. The gradient profiles obtained for ^{3}H-estradiol-17β binding were as follows: ^{3}H-estradiol-17β in the presence of buffer alone (*A*) or 20% DMSO (*B–F*), plus 2 $\times$ 10^{-5} M CN-55,945-27 (*B*), buffer (*C*), 1 $\times$ 10^{-4} M phenester (*D*), 1 $\times$ 10^{-4} M estradiol mustard (*E*), and 1 $\times$ 10^{-5} M estradiol mustard (*F*). Using the computer program of Brooks and Wittliff (12), the area under the curve for the 8S peak was determined and expressed as percentage of the total radioactivity applied to the gradient: (*A*) 52%; (*B*) 12%; (*C*) 49%; (*D*) 52%; (*E*) 20%; and (*F*) 38%.

[9] Sherman MR, Atienza SBP, Shansky JR, et al. Progesterone receptors of chick oviduct. Steroid-binding "subunit" formed with divalent cations. Submitted to the *Journal of Biological Chemistry*, 1974.

TABLE 1.—Time course of the effect of estradiol mustard on the binding of ^{3}H-estradiol-17β to receptors in rat uterine cytosol*

Time (hrs)	^{3}H-Estradiol-17β specific binding		Inhibition of binding by estradiol mustard (%)
	Alone†	In presence of 1×10^{-5} M estradiol mustard†	
1	86305 ± 1946 (4)	67614 ± 1442 (4)	22
3	96392 ± 241 (4)	75611 ± 1286 (4)	22
5	98685 ± 1430 (3)	73833 ± 2772 (4)	25
13.6	97440 ± 411 (3)	74363 ± 3090 (4)	24

*Aliquots of rat uterine cytosol (0.2 ml) were incubated at 4 C with 4.5 nM ^{3}H-estradiol-17β (114 curies/mM) in the presence or absence of 1×10^{-5} M estradiol mustard and specifically bound ^{3}H-estradiol-17β was determined at the end of the incubation period using dextran-coated charcoal as described in Materials and Methods.

†Figures indicated are the mean ± SE with the number of samples indicated in parentheses.

resulting from the breakdown of estradiol mustard. The results are presented in table 1. The specific binding of ^{3}H-estradiol-17β to receptor sites in the uninhibited samples was maximal after 3 hours and remained unchanged for the duration of the incubation period. The inhibition of specific binding by 1×10^{-5} M estradiol mustard was not significantly different (range, 22%–25%) over the incubation period.

DISCUSSION

It is currently accepted that an essential characteristic of steroid hormone-responsive cells is the ability of the hormone to bind specifically to sites on proteins in the cytoplasm that are of high affinity but limited capacity. These "receptors" are then transferred by a sequential process to the nucleus where the ultimate effect of the hormone is expressed. Recent reports indicate that hormone antagonists block the action of natural hormones such as estrogens (11) or glucocorticoids (13). They can bring about these effects in one of two ways: (*a*) by competing with the natural hormone for uptake into the cell and thus preventing hormone entry, or (*b*) by preventing the binding of the hormone to its characteristic receptor. As steroid hormones enter their target cells, apparently by passive diffusion, the effect of antagonists would not be expected at the level of the plasma membrane. Accordingly, our studies with estradiol mustard, an estrogen analog (1,3), were carried out in vitro to determine the effect of this agent on the binding of the estradiol-17β to its cytosol receptor from rat uteri. The results of this study are in accord with those published by others using estrogen antagonists such as ethamoxytriphetol, clomiphene, CN-55,945-27, or nafoxidine (11). Estradiol mustard inhibits the binding of 5 nM estradiol-17β to its receptor at greater than 10^{-6} M. Estradiol mustard does not have as high an affinity for the estrogen receptor (ie, Ka $\sim$ 0.3 μM^{-1}) as some estrogen analogs or antagonists (7, 11). However, one selective advantage of estradiol mustard may be that the phenester moiety can interact chemically with the receptor maintaining the antagonist-receptor complex for a longer period of time. This maintenance of antagonist-receptor complex may further block the action of estradiol-17β by preventing the recycling of receptor as postulated by Munck et al (14).

It is possible that the inhibition of ^{3}H-estradiol-17β binding in vitro by estradiol mustard is the result of chemical or enzymatic hydrolysis of the estradiol mustard to free estradiol-17β and phenester. However, the results presented in table 1 do not support this conclusion since one would expect the cleavage of estradiol mustard to be time dependent. Studies currently underway with doubly labeled estradiol mustard indicate that it is the estradiol mustard and *not* free estradiol-17β liberated from the breakdown of the antagonist that is responsible for

the inhibition of binding.[10] Using the labeled estradiol mustard it will be possible to determine whether the mechanism of action of estradiol mustard on estrogen-target cells is similar to that reported by Turnell et al (13) for the antiglucocorticoid, cortexolone, on thymocytes.

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[10] Separation of uterine cytosol previously incubated with chromatographically purified $^{14}C/^{3}H$-labeled estradiol mustard ([6,7-^{3}H]-estra-1,3,5(10)-triene-3,17β-diol, bis[[p-[bis(2-chloroethyl-[1,2-^{14}C]-amino]phenyl]-acetate]) by centrifugation on linear sucrose gradients gave specific superimposable carbon-14 and tritium peaks.

Preliminary Report on the Treatment of Nonresectable Cancer of the Lung [1,2,3]

D. T. Kaung, [4] J. Wolf, [5] L. Hyde, [6] and M. Zelen [7]

Veterans' Administration Lung Cancer Study Group

SUMMARY

Between April 1969 and March 1972, 1546 patients who had nonresectable bronchogenic carcinoma received treatment according to the Veterans' Administration Lung Cancer Study Group protocols. Two hundred and thirty-nine of these patients had tumors confined to one hemithorax and were amenable to local supravoltage radiotherapy, chemotherapy, or both. Preliminary results indicated no definite overall superiority of one regimen over the others. Thirteen hundred and seven patients who had more extensive tumor involvement were treated with regimens consisting of high and low doses of L-asparaginase, NSC-45388, dibromodulcitol, hexamethylmelamine, procarbazine, CCNU, and streptozotocin. The results based on survival were compared with those of the concurrent standard reference regimen of intravenous cyclophosphamide. None of the newer regimens appeared to be superior to cyclophosphamide. Analysis of the background variables of patients indicated that initial performance status had a consistently strong influence on prognosis in the extensive disease group.

[Cancer Chemother Rep 58:359–364, 1974]

Since its inception in 1958, the Veterans' Administration Lung Cancer Study Group has studied a large number of chemotherapeutic agents, as well as radiotherapy, in the treatment of patients with nonresectable lung can-

[1] Received July 31, 1973; revised Nov 9, 1973; accepted Nov 19, 1973.

[2] This study was done under the auspices of the Research Service, Veterans' Administration Central Office, and the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), by the following participating investigators: *Joseph B. Mizgard*, Ann Arbor, Mich; *Richard W. Dwight* and *Robert M. Amick*, Boston, Mass; *Julius Wolf* and *Herbert E. Edlis*, Bronx, NY; *Sidney Sbar* and *Seymour Olshin*, Brooklyn, NY; *Richard M. Whittington* and *Levona W. Olmsted*, Cleveland, Ohio; *Hisashi Majima*, East Orange, NJ; *David T. Kaung*, Iowa City, Iowa; *Leroy Hyde*, Long Beach, Calif; *Albert Fink*, Los Angeles, Calif; *James Yee*, Martinez, Calif; *Frank S. Dietrich* and *John P. Griffin*, Memphis, Tenn; *Edward W. Humphrey*, Minneapolis, Minn; *Clyde O. Brindley*, Mountain Home, Tenn; *Stewart M. Scott* and *Frank K. Justice*, Oteen, NC; *Henry P. Close*, *Stewart McCracken*, and *Ralph M. Myerson*, Philadelphia, Pa; *John L. Fairly, Jr.*, Richmond, Va; and *Lyndon E. Lee, Jr.* (coordinator), Washington, DC.

[3] Drugs used in this study were supplied by DCT, NCI. Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are listed in the List of Compounds at the end of the text.

[4] Medical Service, Veterans' Administration Hospital (VAH), and Department of Internal Medicine, University of Iowa College of Medicine, Iowa City.

[5] Medical Service, VAH, Bronx, NY.

[6] Pulmonary Service, VAH, Long Beach, Calif.

[7] Statistical Laboratory, State University of New York at Buffalo, Amherst.

cer. Up to the present time, 13 protocols of controlled clinical trials have been used in sequence. This report outlines the preliminary results obtained from protocols 10, 11, and 12. Detailed accounts of various aspects of these studies are in preparation.

The general design of the study has been presented previously (1). All patients in the participating hospitals who had histologically or cytologically proven nonresectable bronchogenic carcinoma were admitted to the study. On admission, the patients were divided into limited disease groups and extensive disease groups according to the degree of their disease. In the limited disease group the tumor must be confined to one hemithorax. Local extension or nonpalpable positive ipsilateral supraclavicular nodes may be present if the entire tumor can be adequately treated by irradiation. All patients who did not meet the specific criteria for the limited disease group were treated in the extensive disease group. Previous studies from the Veterans' Administration Group had indicated the desirability of separating patients into the two groups during the evaluation of therapy (2–4).

In this study we used the percent of survival over time as the primary criterion for assaying effectiveness of treatment. Although randomization and large sample size tend to minimize intergroup differences, detailed analyses of background variables showed that the general clinical status and cell type were important factors affecting patients' responses to treatment.

TREATMENT REGIMENS

Protocol 10—initiated in April 1969

Limited disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Supravoltage radiotherapy, 4000–5000 rads in 4–6 weeks;

b. Cyclophosphamide, 0.3 g/m²/ day intravenously (iv) × 5, followed by 200 mg/day orally;

c. Supravoltage radiotherapy, 4000–5000 rads in 4–6 weeks, plus cyclophosphamide, 200 mg/day orally.

Extensive disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Cyclophosphamide, 1.1 g/m² iv every 3 weeks;

b. NSC–45388, 325 mg/m²/day iv × 5 (repeat course every 26 days);

c. High-dose L-asparaginase, 1500 IU/kg/wk iv × 6;

d. Low-dose L-asparaginase, 150 IU/kg/wk iv × 6;

e. Dibromodulcitol, 400 mg/m²/wk orally × 6.

Protocol 11—initiated in February 1970

Limited disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Supravoltage radiotherapy, same as protocol 10;

b. BCNU, 185 mg/m² iv every 6 weeks.

Extensive disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Cyclophosphamide, same as protocol 10;

b. Dibromodulcitol, same as protocol 10;

c. Low-dose L-asparaginase, 150 IU/kg/wk iv × 6.

Protocol 12—initiated in June 1970

Limited disease group.—Same as protocol 11.

Extensive disease group.—Patients were randomly assigned to one of the following treatment regimens:

a. Cyclophosphamide, same as protocol 10;

b. L-Asparaginase, same as protocol 11;

c. CCNU, 130 mg/m² every 6–8 weeks;

d. Procarbazine, 150 mg/m²/day orally;

e. Hexamethylmelamine, 8 mg/kg/day orally;

f. Streptozotocin, 1 g/m²/wk iv × 10.

RESULTS

Limited disease group.—The median survival times of patients treated according to protocols 10 and 11 are shown in table 1. Cyclophosphamide or radiotherapy given alone appeared to be as effective as cyclophosphamide plus radiotherapy given in combination. BCNU also compared favorably with radiotherapy. When only squamous-cell carcinomas are con-

sidered, the radiotherapy group shows a significantly longer median survival time (60 ± 11.6 weeks) than the BCNU-treated group (25 ± 5.1 weeks). Since 52.5% of patients with well-differentiated squamous-cell carcinomas die with tumors still limited to the chest,[8] the data suggest that local irradiation is more effective than chemotherapy for this cell type. However, a similar group of patients who had squamous-cell carcinoma treated with the same radiotherapy regimen as in protocol 10 showed a median survival time of only 18.3 weeks, thus indicating the important influence that factors other than modalities of treatment have on survival. Similarly, although BCNU appeared to be more effective than radiotherapy for the cell type designated as "others," the histologic heterogeneity of the group and the relatively small number of patients studied preclude any meaningful interpretation.

Extensive disease group.—In this group of patients there was a consistent linear correla-

[8] Personal communication from Dr. Raymond Yesner, based on data from the Veterans' Administration Lung Group.

TABLE 1.—Median survival time (wks) by treatment and cell type: limited disease

		Protocol 10		Protocol 11	
Cell type	Treatment	Median survival time	Total No. of deaths/ total No. of patients	Median survival time	Total No. of deaths/ total No. of patients
All types	Cyclophosphamide	25.7 (5.7)*	20/23	27.4 (3.08)	79/89
	Radiotherapy	20.5 (4.5)	21/22		
	Radiotherapy + cyclophosphamide	21.8 (4.5)	23/25	27.9 (3.36)	69/80
	BCNU				
Squamous-cell carcinoma	Cyclophosphamide	10.0	8/9		
	Radiotherapy	18.3	10/10	60.1	27/32
	Radiotherapy + cyclophosphamide	33.7	8/9		
	BCNU			25.0	24/27
Small cell carcinoma	Cyclophosphamide	28.9	5/7		
	Radiotherapy	21.6	4/4	22.0	13/14
	Radiotherapy + cyclophosphamide	20.8	4/4		
	BCNU			27.9	18/20
Adeno-carcinoma	Cyclophosphamide	23.0	2/2		
	Radiotherapy	—	0/0	17.4	9/9
	Radiotherapy + cyclophosphamide	20.3	3/3		
	BCNU			22.0	9/10
Large cell carcinoma	Cyclophosphamide	29.8	1/1		
	Radiotherapy	13.6	3/3	32.7	11/13
	Radiotherapy + cyclophosphamide	—	0/0		
	BCNU			35.3	5/7
Others†	Cyclophosphamide	36.5	4/4		
	Radiotherapy	20.8	4/5	23.0	19/21
	Radiotherapy + cyclophosphamide	20.8	8/9		
	BCNU			59.2	13/16

*Standard deviation in parentheses.

†Disease confirmed by cytology only, or cases not easily classified.

TABLE 2.—Median survival time (wks) by treatment and cell type: extensive disease

Cell type	Treatment	Protocol 10		Protocol 11		Protocol 12	
		Median survival time	Total No. of deaths/ total No. of patients	Median survival time	Total No. of deaths/ total No. of patients	Median survival time	Total No. of deaths/ total No. of patients
All types	Cyclophosphamide	15.4 (1.6)*	96/101	11.7 (1.3)	78/81	13.1 (0.93)	200/212
	L-Asp (L)†	15.9 (2.6)	38/38	8.5 (0.9)	85/86	5.8 (0.9)	47/49
	L-Asp (H)†	4.3 (0.9)	23/24	—	—	—	—
	NSC-45388	10.0 (1.0)	95/100	—	—	—	—
	Dibromodulcitol	8.7 (1.0)	71/82	11.2 (1.8)	40/42	—	—
	CCNU	—	—	—	—	11.7 (1.3)	78/81
	Hexamethylmelamine	—	—	—	—	9.6 (1.1)	79/83
	Streptozotocin	—	—	—	—	9.6 (0.9)	115/124
	Procarbazine	—	—	—	—	10.0 (0.74)	182/194
Squamous-cell carcinoma	Cyclophosphamide	20.6	20/22	11.1	15/16	15.5	53/58
	L-Asp (L)	27.1	7/7	11.7	30/30	5.6	14/14
	L-Asp (H)	2.4	5/5	—	—	—	—
	NSC-45388	5.9	21/21	—	—	—	—
	Dibromodulcitol	7.8	16/18	6.1	8/8	—	—
	CCNU	—	—	—	—	11.6	22/22
	Hexamethylmelamine	—	—	—	—	8.1	25/25
	Streptozotocin	—	—	—	—	9.9	27/30
	Procarbazine	—	—	—	—	14.3	29/34
Small cell carcinoma	Cyclophosphamide	14.8	14/14	6.4	10/10	12.2	33/34
	L-Asp (L)	17.5	4/4	11.6	12/12	3.9	7/8
	L-Asp (H)	4.4	2/2	—	—	—	—
	NSC-45388	9.5	13/13	—	—	—	—
	Dibromodulcitol	4.5	7/8	3.5	5/5	—	—
	CCNU	—	—	—	—	9.2	13/13
	Hexamethylmelamine	—	—	—	—	7.7	17/17
	Streptozotocin	—	—	—	—	5.4	15/15
	Procarbazine	—	—	—	—	7.6	29/29
Adenocarcinoma	Cyclophosphamide	10.1	14/14	13.3	21/21	11.8	25/26
	L-Asp (L)	14.8	6/6	8.4	18/18	4.5	10/11
	L-Asp (H)	7.9	5/5	—	—	—	—
	NSC-45388	7.1	12/13	—	—	—	—
	Dibromodulcitol	5.5	12/12	13.1	8/8	—	—
	CCNU	—	—	—	—	10.8	12/14
	Hexamethylmelamine	—	—	—	—	11.0	5/6
	Streptozotocin	—	—	—	—	12.4	20/21
	Procarbazine	—	—	—	—	12.0	30/31

Large cell carcinoma	Cyclophosphamide	10.6	11/11	11.9	16/17	14.9	33/34
	L-Asp (L)	6.5	7/7	4.6	10/10	6.5	6/6
	L-Asp (H)	2.9	5/5	—	—	—	—
	NSC-45388	14.2	17/17	—	—	—	—
	Dibromodulcitol	11.3	10/11	6.4	5/5	—	—
	CCNU	—	—	—	—	10.2	12/13
	Hexamethylmelamine	—	—	—	—	10.5	15/16
	Streptozotocin	—	—	—	—	9.9	21/22
	Procarbazine	—	—	—	—	8.5	30/32
Others‡	Cyclophosphamide	18.7	37/40	12.5	16/17	12.6	56/60
	L-Asp (L)	8.4	14/14	7.2	15/16	7.7	10/10
	L-Asp (H)	3.9	6/7	—	—	—	—
	NSC-45388	16.5	32/36	—	—	—	—
	Dibromodulcitol	20.1	26/33	13.8	14/16	—	—
	CCNU	—	—	—	—	11.0	19/19
	Hexamethylmelamine	—	—	—	—	12.3	17/19
	Streptozotocin	—	—	—	—	11.6	32/36
	Procarbazine	—	—	—	—	9.3	64/68

*Standard deviation in parentheses.
†L-Asp (L) = low-dose L-asparaginase; L-Asp (H) = high-dose L-asparaginase.
‡Disease confirmed by cytology only, or cases not easily classified.

tion between log survival and the clinical condition of the patients (Karnofsky scale) (5) at the beginning of treatment, regardless of the therapeutic regimen used (6). The median survival times of patients treated with the various regimens are shown in table 2. The standard reference regimen of cyclophosphamide continued to do well when compared with the other treatment regimens. High-dose L-asparaginase was clearly an inferior regimen. Some regimens seemed to be more effective for specific cell types. The significance of the differences and the effects of important concomitant variables such as performance status, weight loss, and other factors on these differences will be discussed in detail in another report.

It is obvious from our studies that stratification according to cell types and extent of tumor involvement plus strict randomization did not insure uniformity of patient populations being compared. This undoubtedly was one of the major factors which caused the median survival time to fluctuate from year to year even when the same treatment regimen was used by the same hospitals. Conclusions based on studies of small numbers of patients (even though statistically significant) must be accepted with caution, since even the best available treatment is only slightly more effective than the others. Of the many patient variables which may affect the results of therapy, initial performance status exerted a consistent influence regardless of the type of therapy in the extensive disease group. This factor was therefore taken into account in protocol 13 in which patients were randomized separately into high- and low-performance status groups.

The overall results of therapy continue to be disappointing. Since combination and sequential therapies have been so successful in acute leukemias, lymphomas, and other solid tumors (7), treatment regimens employing these principles are being tried in protocol 13.

LIST OF COMPOUNDS

L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

Dibromodulcitol: NSC-104800; CAS reg. No. 10318-26-0; galactitol, 1,6-dibromo-1,6-dideoxy-; DBD

Hexamethylmelamine: NSC-13875; CAS reg. No. 645-05-6

NSC-45388: CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Streptozotocin: NSC-85998

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Response and Survival of Patients With Metastatic Neuroblastoma After Combination Chemotherapy With Adriamycin (NSC-123127), Cyclophosphamide (NSC-26271), and Vincristine (NSC-67574) [1,2]

Marco Gasparini, Franca Fossati Bellani, Renato Musumeci, and Gianni Bonadonna [3,4]

SUMMARY

Nineteen consecutively admitted patients who had stage IV neuroblastoma and were previously untreated were given combination chemotherapy with adriamycin, cyclophosphamide, and vincristine. The therapeutic effects and survival rates of these patients are reported. Although no patients achieved a complete remission, six patients achieved a partial response greater than 50% and five patients showed an objective improvement. Responders (11 of 19 or 59%) had a median survival time of 17.5 months and nonresponders had a median survival time of 5 months ($P<0.01$). The median survival time for both responders and nonresponders was 13.2 months. This figure is not statistically significant when compared with a similar group of 23 patients who were treated with single-agent chemotherapy given sequentially (7.5 months).

[Cancer Chemother Rep 58:365–370, 1974]

In the past few years several therapeutic approaches for the management of neuroblastoma have been reported (1–7). All reports advocate the usefulness of surgery (even if not radical), radiotherapy, and chemotherapy. In the apparently localized disease the combination of surgery plus radiotherapy is thought to be an adequate treatment (8–11). Available data suggest that chemotherapy neither increases survival nor decreases mortality (1,6,12,13). Although chemotherapy is the preferred treatment in the advanced and disseminated stages of neuroblastoma, some investigators do not agree on the real usefulness of chemotherapy in all forms of metastatic neuroblastoma (11,14). An adequate evaluation of retroperitoneal nodes, bone marrow, and liver in patients with neuroblastomas (especially those present in children older than 2 years of age) reveals that the majority have metastatic disease (13). Therefore, chemotherapy plays an important role in the different therapeutic approaches.

During the past few years cyclophosphamide and vincristine were found to produce objective responses of 28%–79% in patients when these agents were used singly through different dose schedules (2,4,15,16). The overall therapeutic responses produced by these agents used in combination ranged from 32% to 79% (1,3,6, 7,17). However, the regression obtained with these two drugs administered in sequence and in combination was not translated into an increased overall survival rate that was compa-

[1] Received Aug 2, 1973; revised Dec 4, 1973; accepted Dec 20, 1973.

[2] Cyclophosphamide: CAS reg. No. 6055-19-2; 2*H*-1,-3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; CTX; EDX.

Vincristine: CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1); VCR.

[3] Istituto Nazionale Tumori, Milan, Italy.

[4] *Reprint requests to*: Dr. Gianni Bonadonna, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy.

TABLE 1.—Site of metastases in 19 consecutive patients with neuroblastoma

Primary tumor site	No. of patients	Site of metastases						
		Bone	Bone marrow	Lymph nodes	Skin	Lungs	Liver	Ovaries
Thorax	2	1		1				
Abdomen	12	8	3	10	1	2	1	
Unknown	5	3		2	1			1
Totals	19	12	3	13	2	2	1	1

rable to that achieved in other neoplastic diseases of childhood (12,13).

During phase I and II studies with adriamycin using different dose schedules, prompt regressions were produced in 40%–80% of patients with diffuse neuroblastoma even if these patients were previously treated with conventional agents (18,19). For this reason, in 1971 our Institute began a prospective study which included the combination of adriamycin, cyclophosphamide, and vincristine in consecutive patients with metastatic neuroblastoma. The scope of this paper is to report the preliminary data concerning both responses and survival rates achieved with the above-mentioned combination.

PATIENTS AND METHODS

From January 1971 to December 1972, 19 consecutive patients (ten girls and nine boys) ranging in age from 7 months to 14 years were entered into the study. Seven patients were less than 2 years of age. All patients had diffuse biopsy-proven neuroblastoma that was untreated.

On admission, the extent of disease was documented through the following observations: chest roentgenogram, bone survey, intravenous (iv) urography, lower limb lymphography, liver scan, bone marrow aspiration, estimation of 24-hour urinary excretions of vanilylmandelic acid (VMA) and catecholamines. Abdominal arteriograms were taken for nine of 19 patients. After clinical and radiologic evaluations were conducted, all patients were classified as having stage IV disease. In the present series no patients were classified as stage IV-S (14). The site of metastases is reported in table 1.

Figure 1 outlines the treatment schedule employed in this trial. All drugs were administered by rapid iv injection. Radiation therapy with cobalt was employed only after three cycles of combination chemotherapy. The irradiation was delivered either to the site of initial bulky disease (usually the abdomen) or to one or more symptomatic sites for palliative purposes (eg, painful bone metastases). Tumor dosage ranged from 2000 to 3500 rads. Chemotherapy was usually started 2–4 weeks after diagnosis and was continued until clearcut

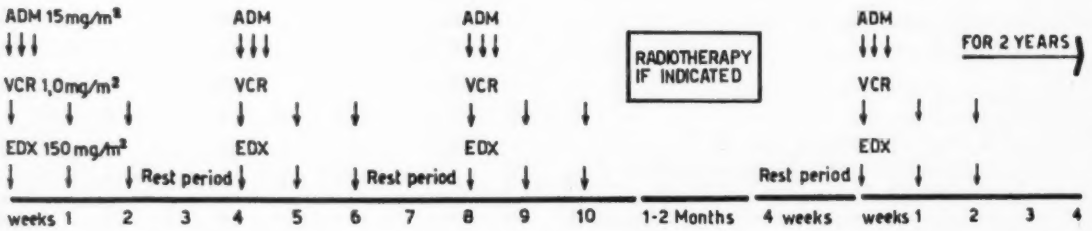

FIGURE 1.—Outline of the treatment schedule. ADM = adriamycin; VCR = vincristine; and EDX = cyclophosphamide.

evidence of disease progression occurred. At this point, either actinomycin D, BCNU, or CCNU was tried.[5]

The criteria for evaluating the degree of drug response were as follows: *complete response* = disappearance of all signs and symptoms of disease including recalcification of osseous metastases and a return to normal levels of VMA and catecholamines; *partial response* = 50% or greater decrease in the product of the longest perpendicular tumor diameters; and *objective improvement* = 25%–50% decrease in the longest perpendicular tumor diameters. Progression was defined as an increase of at least 25% in tumor measurements or the appearance of new lesions even in the presence of regression elsewhere.

Survival time from the beginning of therapy was calculated by the actuarial method. A similar analysis was conducted in 23 consecutive patients with diffuse neuroblastoma treated with single-agent chemotherapy given sequentially (cyclophosphamide, vincristine, actinomycin D, daunorubicin,[6] and adriamycin) from 1968 to 1970.

RESULTS

Table 2 reports the response to combination chemotherapy in relation to the site of primary and metastatic lesions. No patients achieved a complete remission in all parameters. Six patients showed a partial regression greater than 50%, and five patients showed a 25%–50% objective improvement. From this limited number of patients, only the abdominal, osseous, and nodal lesions can be evaluated. The primary abdominal tumor responded in 66% of the cases and six of 12 regressions were classified as greater than 50%. Response of the metastatic lymph nodes was observed in 38% of patients and in two patients a complete regression was obtained. In no patients was a recalcification of bone metastases observed. This is the main reason why no patients were classified as complete responders.

In responsive patients the initial regression occurred after the first cycle of treatment, and in a large majority of cases the maximum regression was achieved after the third cycle of treatment. The median duration of regression was 11 months (range: 2.5–20 months). At the time of this report, six of 11 responders are still alive while only one of eight nonresponders is alive. In responsive patients death occurred after a median time of 17 months (range: 9–20 months) from beginning of therapy, whereas death occurred in the unresponsive patients after a median time of 5 months (range: 2–13 months). Four of seven patients less than 2 years of age showed no response to treatment and their deaths occurred after a median time of 4 months.

VMA determination showed abnormal levels in a total of 12 of 19 patients (63%). In

[5] Actinomycin D: NSC-3053.

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

[6] Daunorubicin: NSC-83142; CAS reg. No. 23541-50-6.

TABLE 2.—Response to chemotherapy in relation to site of lesions

Primary tumor site	No. of patients	No. of patients with response				Overall regression
		None	25%–50%	> 50%	CR*	
Thorax	2	1	1			50%
Abdomen	12	4	1	6	1	66%
Bone	12	12				0
Bone marrow	3	1			2	66%
Lymph nodes†	13	8	2	1	2	38%
Skin	2			1	1	0
Lung	2	2				0
Liver	1	1				0

*CR = complete remission.
†Peripheral and retroperitoneal.

unresponsive patients the levels of VMA remained essentially unchanged, whereas in all six responsive patients the abnormal levels of VMA decreased to normal.

In our group of patients treated sequentially with single-agent chemotherapy the median survival of responders was 9.2 months as compared with 4.3 months for nonresponders ($P < 0.01$).

Figure 2 reports the actuarial analysis of the survival time of responsive versus unresponsive patients. The median survival time of responsive patients (17.5 months) is more than three times longer than that of unresponsive patients (5 months). This difference is statistically significant ($P < 0.001$). By subtracting the time spent in remission, the survival time of responders becomes similar to that of nonresponders.

Figure 3 compares the overall actuarial survival time of all patients treated with combination chemotherapy (13.2 months) with the survival time of patients treated with single-agent chemotherapy given in sequence (7.5 months). There is a difference of 5 months in the median survival time between the two groups that are similar in terms of age and extent of disease. However, this difference is not statistically significant ($P < 0.5$).

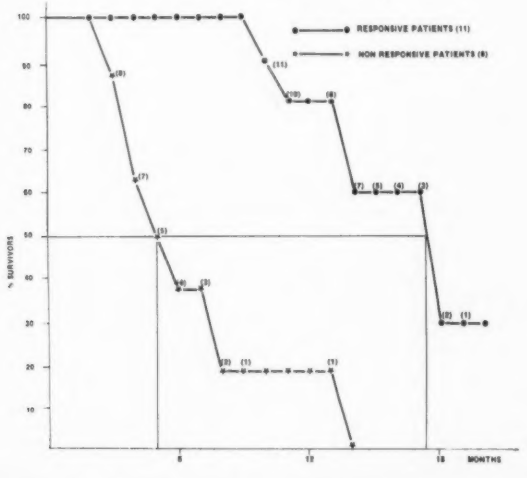

FIGURE 2.—Survival after combination chemotherapy with adriamycin, cyclophosphamide, and vincristine. In parentheses are the numbers of survivors.

Combination chemotherapy was usually well tolerated by the patients in this study. The most frequent toxic manifestation was alopecia which occurred in 15 of 19 (80%) patients. Leukopenia with a white blood cell (wbc) count that fell below 3000 cells/mm^3 occurred in all patients; however, only in one case was there a wbc count below 1000 cells/mm^3. Thrombocytopenia was always moderate, and in no instance did the platelet count fall below 50,000 platelets/mm^3. Bone marrow depression usually recovered during the interval between treatment cycles, so only occasionally was there a delay in continuing therapy. Nausea and vomiting, stomatitis, constipation, and paresthesia were always mild and never required a delay or a dose reduction between treatment cycles. There was no clinical, radiologic, or electrocardiographic evidence of cardiomyopathy. The mean total dose of adriamycin administered in this study was 260 mg/m^2 (range: 35–600 mg/m^2). Upon disease progression patients have failed to respond to subsequent treatment with either nitrosoureas or actinomycin D or both.

COMMENT

In the past decade there has been no substantial improvement in the management of neuroblastoma. Since the diagnosis is usually made after the neoplasia has become clinically obvious, neuroblastoma remains a difficult disease to cure (especially in children older than 2 years of age). Metastatic disease is also difficult to control beyond the sixth month from the beginning of therapy. With cyclophosphamide and vincristine given in combination, the median survival time from diagnosis was 6 months in the series reported by Sutow et al (12), 8 months in those series reported by Sawitsky et al (5), and 13 months in the series reported by Pinkel et al (1). Recently, Helson et al (7) have reported an overall response rate of 60% and a median survival time of 17 months from diagnosis by combining cyclophosphamide, vincristine, and daunorubicin administered after surgery plus radiotherapy. Although the dose of daunorubicin in Helson's series is equal in terms of mg/m^2 to the dose of adriamycin in

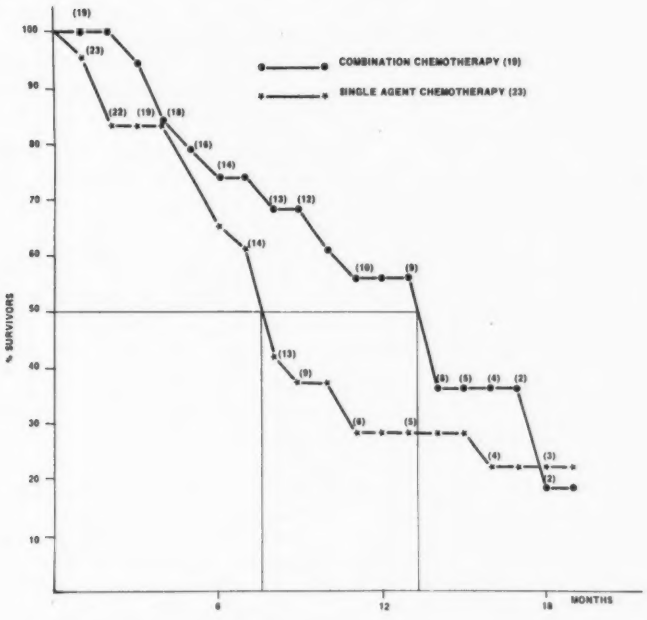

FIGURE 3.—Comparison of survival after combination chemotherapy (19 patients) and single-agent chemotherapy (23 patients). In parentheses are the numbers of survivors.

our series, the drug schedule as well as the doses of vincristine and especially of cyclophosphamide are different in the two therapeutic regimens. These successful and beneficial responses which could be partially attributed to the introduction of daunorubicin were long-lasting only in the patients under 2 years of age (nine of 20) at diagnosis.

In our series combination chemotherapy yielded a significantly increased survival rate in responders compared with that of nonresponders. This improved survival rate was not obtained through the achievement of complete remissions, since osseous recalcification was never observed in our group of patients. However, the fact that the subtraction of the time spent in remission from the group of responders yields a survival curve similar to that of nonresponders, definitely proves that combination chemotherapy was able to favorably affect the survival time. Radiation therapy which was not given to all patients did not contribute substantially to the increased survival time in responsive patients because it was delivered through limited fields and given mainly for palliative purposes. Although the number of children in this study is still too small to allow definitive conclusions, there is no particular difference within the group of responders in terms of sex and age distribution, site of primary tumor, and pattern of metastatic disease. The comparison of the survival curve of the whole group treated with combination chemotherapy to that of the group given single-agent chemotherapy fails to show in retrospect a statistically significant difference in the median survival time. Therefore, despite the limits of historical comparisons, we can not state on the basis of the available data that combination chemotherapy is superior to single-agent chemotherapy in terms of an increase in overall survival time and a decrease in percent of mortality. Although a more definitive answer for this particular point can be provided only through prospective controlled studies, our experience shows that combination chemo-

therapy which includes adriamycin has proved to be useful in controlling neuroblastomas in responsive patients.

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Effect of Hexamethylmelamine (NSC-13875) on Small Cell Carcinoma of the Lung (Phase II Study) [1,2]

Hiroshi Takita and Mukund S. Didolkar [3]

SUMMARY

The results of a phase II clinical trial of hexamethylmelamine in 23 patients with small cell carcinoma of the lung are presented. The overall response rate was 43%. The median survival time was 5.5 months and the mean survival time was 8.4 months. The most frequent side effects were nausea and vomiting; the hematopoietic toxicity was rather mild and transient. Because of relatively mild side effects, hexamethylmelamine should be further evaluated for treatment of small cell carcinoma in combination with other chemotherapeutic agents or with radiation therapy.

[Cancer Chemother Rep 58:371–374, 1974]

Hexamethylmelamine is one of the substituted melamines derived from cyanuric chloride. It was originally investigated for possible use in synthetic resin and synthetic fibers (1). Subsequently, an antitumor effect of hexamethylmelamine against Sarcoma 180 in mice was detected when ethylenimine derivatives and related compounds were screened in this tumor system (2). A phase I study conducted in 1965 showed effectiveness of hexamethylmelamine against carcinoma of the lung and ovary (3–5).

The chemical structure of hexamethylmelamine resembles that of the anticancer drug triethylenemelamine,[4] however, it has been suggested that hexamethylmelamine may not function as an alkylating agent but perhaps as a pyrimidine antimetabolite (6).

Our previous clinical study of this drug for various cell types of bronchogenic carcinoma revealed promising effectiveness against small cell carcinoma (7). The results of our extended phase II clinical study of hexamethylmelamine in 23 patients with small cell carcinoma of the lung are analyzed.

MATERIALS AND METHODS

The micronized form of hexamethylmelamine was supplied in capsules containing 100 mg of the drug. In most cases the total dosage was 12 mg/kg/day for 21 days administered orally in equal doses three times a day 1½–2 hours after meals. Prochlorperazine (Compazine) or chlorpromazine (Thorazine) was usually prescribed.

The following criteria were used in the selection of patients to be treated with hexamethylmelamine:

1. Histologically confirmed small cell carcinoma of the lung.
2. Ambulatory or semiambulatory at the start of the study.
3. White blood cell count (wbc) of 5000 cells/mm^3 or higher and platelet count of 100,000 cells/mm^3 or higher.
4. No radiation therapy or chemotherapy for at least 4 weeks.

All patients were periodically examined.

[1] Received Aug 21, 1973; revised Jan 7, 1974; accepted Jan 24, 1974.

[2] Hexamethylmelamine: CAS reg. No. 645-05-6. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute.

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[4] Triethylenemelamine: NSC-9706; CAS reg. No. 51-18-3; *s*-triazine, 2,4,6-tris(1-aziridinyl)-.

Chest roentgenograms, liver scans, blood cell counts, and blood chemistry tests were performed at regular intervals.

The course of hexamethylmelamine was repeated at intervals of 6–12 weeks. Twenty-three patients were treated with one or more courses of the drug. In addition, four patients received a course of radiation therapy (3000 rads) to the primary lesion and the regional lymph node area, and one patient received radiation therapy for brain metastases.

RESULTS

Toxicity

Gastrointestinal symptoms in the form of nausea, vomiting, and anorexia were common and were observed in 15 patients (71%). Antiemetics usually controlled the symptoms, but in one case hospitalization was necessary to continue administration of hexamethylmelamine.

Leukopenia (3000–1000 cells/mm^3) was observed in eight patients. A gradual decrease in the wbc was noted 1 week after the start of the medication. The wbc reached its nadir at the third or fourth week after the start of therapy and was back to normal by the sixth week.

In two patients, the platelet count dropped below 100,000 cells/mm^3 (75,000 and 68,000 cells/mm^3) in the fourth week, but the counts returned to normal by the sixth week.

Up to nine courses of hexamethylmelamine were given but no cumulative toxic effects were observed. There were no neurologic symptoms or death due to the drug toxicity. No toxicity to the liver or kidney was observed.

Antitumor Effect (Table 1)

Of 23 patients, 21 had measurable lesions for evaluation of the response rate. Overall response rate (> 50% regression of the lesions) was 43% (nine patients). Three patients had a complete regression of the lesion and six patients had a greater than 50% regression of the lesion. Transient and less than 50% regression of the lesion was observed in two patients. There were ten nonresponders.

In this series the median survival time was 5.5 months (from the start of the therapy) and the mean survival time was 8.4 months.

Extent of Disease and Response Rate (Table 2)

There were five responders among ten patients with the disease localized in the thorax, and four of 11 patients with disseminated disease responded to hexamethylmelamine. In almost all cases, primary lesions as well as any metastatic lesions responded equally to hexamethylmelamine.

Previous Treatment and Response Rate

Three patients had previously received radiation therapy, two of whom responded. Among these three patients, there was one responder to hexamethylmelamine who also had previously responded to radiation therapy. Two patients had previously received cyclophosphamide [5] and both of them responded. One of these two patients responded to hexamethylmelamine.

Subsequent Treatment

Two of the nonresponders to hexamethylmelamine have subsequently received adriamycin (NSC-123127) and responded. They survived 13 and 14 months respectively (omitted from computation of overall survival).

DISCUSSION

Small cell carcinoma of the lung is known to carry a grave prognosis, and the overall 5-year

[5] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

TABLE 1.—Effect of hexamethylmelamine on small cell carcinoma (total No. of patients, 23)

No. of responding patients*				
100% regression	> 50% regression	< 50% regression	Regression not measurable	No. of unresponsive patients
3	6	2	2	10

*Response rate = 43%.

TABLE 2.—Effect of hexamethylmelamine on small cell carcinoma: extent of disease and response

Patient No.	Location of response in patients responding		
	100% regression	> 50% regression	< 50% regression
1	Primary, mediastinal nodes		
2	Primary, liver		
3	Primary, mediastinal nodes, and abdominal mass		
4		Primary, liver*	
5		Primary, mediastinal nodes	
6		Primary, liver	
7		Primary, mediastinal nodes	
8		Primary, mediastinal nodes	
9		Primary, supraclavicular nodes	
10			Primary, pleural effusion
11†			Skin

*Jaundice and ascites disappeared.

†Recurrence occurred after pneumonectomy. Mediastinal nodes and liver did not respond.

survival rate has been about 1% (8). At the same time, the disease is susceptible to various chemotherapeutic agents (9).

The overall median survival time of 161 patients treated at the Roswell Park Memorial Institute was 4.5 months (8), while there was an improvement in the median survival time of the patients who were treated with hexamethylmelamine (5.5 months from the start of therapy).

The response rate of small cell carcinoma in this series was 43%, which is in agreement with the report of compiled data of the National Cancer Institute; their data show a response rate of 36% among 67 patients with small cell carcinoma (10).

It is of interest that our previous study on the effect of CCNU [6] in 50 patients with carcinoma of the lung showed that none of the patients with disseminated disease (hematogenous metastasis) responded to the drug (11); on the contrary, four of 11 patients with hematogenous metastasis responded to hexamethylmelamine.

The most commonly observed side effects of the drug were nausea and vomiting (71%), while effects on the bone marrow were rather mild. According to the literature, hexamethylmelamine has been given in doses of 4–15 mg/kg/day for 21–90 days, but 12 mg/kg/day has been reported to be the maximally tolerated dose due to nausea and vomiting (3).

It is our opinion that one course given over 21 days is adequate in the treatment of small cell carcinoma since in all of our responders we observed regression of the lesion by the end of 21 days. It appears that the optimal interval of the course is 6–7 weeks since the length of

[6] CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-.

remission after a course of therapy in some of the responders was about 8 weeks.

As a single drug, hexamethylmelamine was found to be effective against small cell carcinoma of the lung, and because of relatively mild hematopoietic toxicity the drug should be further evaluated for treatment of small cell carcinoma in combination with other chemotherapeutic agents or with radiation therapy.

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Renal Dysfunction After Treatment With Isophosphamide (NSC-109724) [1,2,3]

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SUMMARY

After treatment with isophosphamide (5 g/m²), acute renal tubular acidosis, nephrogenic diabetes insipidus, phosphaturia, glucosuria, ketonuria, and generalized aminoaciduria developed in a patient with breast carcinoma. These findings represent drug-induced adult Fanconi syndrome. Nonselective proteinuria reaching the nephrotic range was also observed. The tubular and glomerular abnormalities normalized over several weeks, except for the persistence of a mild tubular acidification defect, vasopressin-fast concentrating defect, and mild proteinuria. Re-treatment of the patient with a lower dose of isophosphamide resulted in the reappearance of the major features of the syndrome. The effects of the two courses of therapy on tubular and glomerular function were not additive with a time interval between doses of 6 weeks.

[Cancer Chemother Rep 58:375–382, 1974]

Isophosphamide is a derivative of cyclophosphamide and has been shown to be effective against animal tumor systems resistant to cyclophosphamide and a variety of advanced malignancies in man (1). In high doses (50–80 mg/kg of body weight) this alkylating agent has resulted in azotemia, hematuria, and urinary frequency (1). In this report we describe a number of renal tubular defects, including aminoaciduria, glucosuria, renal tubular acidosis, and vasopressin-fast concentrating defect, that occurred after treatment with isophosphamide. Generalized tubular defects have not been reported after administration of isophosphamide or other alkylating agents.

CASE HISTORY

A 48-year-old woman with no family history of renal disease was admitted to the Oncology Division, Department of Medicine, Johns Hopkins University, for additional chemotherapy for advanced breast carcinoma. Twenty-six months before this admission, right radical mas-

[1] Received Sept 13, 1973; revised Nov 28, 1973; accepted Jan 8, 1974.

[2] Supported in part by Public Health Service grant CA-06973 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[3] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text. Isophosphamide was supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

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[7] The authors wish to thank Dr. Albert H. Owens, Jr., for his criticism of this manuscript.

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tectomy and bilateral oophorectomy had been performed. Fourteen months later, multiple tumor nodules appeared over the right anterior chest wall and upper arm and were treated with local radiotherapy. Tumor involvement of the left chest wall, left breast, and bilateral neck and supraclavicular nodes was apparent 16 months after diagnosis. The patient had a poor tumor response to combination chemotherapy [9] (8 weeks' duration) and to two courses of cytosine arabinoside (50 mg/kg of body weight, administered intravenously [iv] over 48 hrs). The latter treatment was given 12 weeks before the isophosphamide treatment. At that time renal function was normal: serum urea nitrogen (SUN) ranged from 6 to 15 mg/100 ml, serum creatinine was 0.6–1.0 mg/100 ml, and routine urinalysis (six times) revealed no cellular elements. Urine pH levels ranged from 5 to 6 (Labstix, Ames) and urine specific gravities were as high as 1.019–1.023. Qualitative testing revealed no glucosuria, ketonuria, or proteinuria. Serum chloride was 103–105 mEq/liter, bicarbonate was 25–27 mEq/liter, calcium was 9.0 mg/100 ml, phosphorus was 3.8 mg/100 ml, uric acid was 5.4 mg/100 ml, and serum albumin was 3.6 g/100 ml. Fasting serum glucose was 96–110 mg/100 ml.

Physical examination at the time of admission to our clinic showed a moderately obese, middle-aged, black woman with massive edema of both upper arms. Blood pressure was 150/80 mm Hg, heart rate was 80/minute, respirations were 15/minute, and oral temperature was 98.6 F. Multiple tumor nodules were present over the anterior chest wall with readily apparent bilateral, matted axillary, supraclavicular, and cervical node enlargement. The right breast was absent. There were ulcerating tumor nodules in the left breast. The remaining physical examination results were normal.

Initial laboratory data revealed normal renal function except for a 24-hour urinary protein excretion of 356 mg/day. SUN was 7 mg/100 ml, serum creatinine was 1.0 mg/100 ml, and creatinine clearance was 113 ml/minute. There were no abnormalities of urine sediment; glucosuria and ketonuria were absent and urine pH ranged from 5.10 to 6.12 (samples collected under mineral oil, pH measured with a Beckman Expandomatic pH meter). Twenty-four-hour urinary excretion of potassium, phosphorus, and calcium were within normal limits (fig 1). Urine uric acid excretion was elevated (1010 mg/24 hrs).

METHODS

Clinical data.—Complete 24-hour urine collections, except as noted, were made throughout the hospitalization. In the first course isophosphamide was administered iv over 1 hour at a dose of 5 g/m² (total dose, 9.5 g); a second course of 6.5 g (total dose) was carried out 34 days after the initial treatment. During the initial treatment period, allopurinol (300 mg/day) was administered with isophosphamide. Allopurinol is not known to have a primary

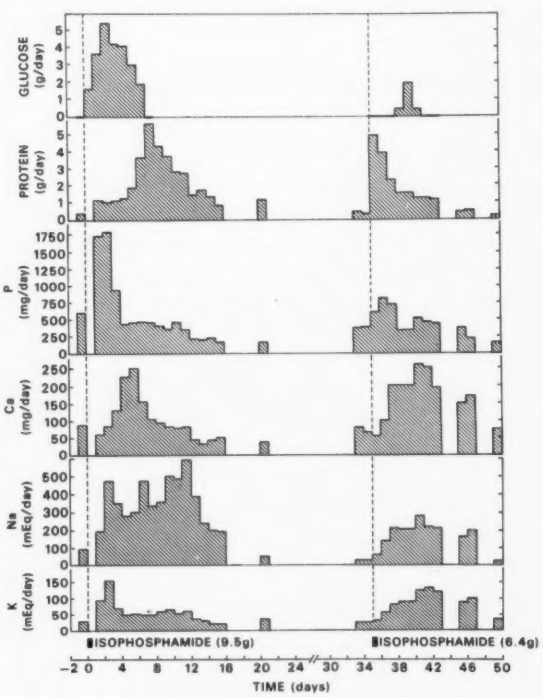

FIGURE 1.—Time sequence of changes during urinary excretion of potassium, sodium, calcium, phosphorus, protein, and glucose after treatment with isophosphamide.

[9] Prednisone, 30 mg/day orally; cyclophosphamide, 50 mg/day orally; 5-fluorouracil, 500 mg/week iv; methotrexate, 25 mg/week iv; and vincristine, 1 mg/week iv.

effect on renal function (2). In the second treatment course, the patient received no drugs in addition to isophosphamide.

Urine and serum electrolytes; urine glucose, ketones, and amino acids.—Sodium, potassium, chloride, bicarbonate, calcium, and phosphorus levels were measured on a Technicon AutoAnalyzer. Urine glucose was measured qualitatively (glucose oxidase, Labstix, Ames) and quantitatively (ferric chloride reagent). Urine and serum ketones were determined with Ketostix (Ames). Qualitative urinary amino acid analyses were performed on samples of selected 24-hour collections (Gilson high-voltage electrophoresis). Quantitative determinations of certain amino acids were also made (3).

Serum and urine protein determinations.—Serum total protein was measured by the method of Gornall et al (4), and urine total protein was measured by the method of Savory et al (5) with bovine albumin as the standard (6). Cellulose acetate membrane electrophoresis of serum and urine was carried out by the method of Laurell et al (7). Strips were stained with ponceau S in 5% trichloroacetic acid and cleared. Densitometry was performed using a Densicord 542 (Photovolt Corp., New York, NY). Immunoelectrophoresis was done in 1.5% Ionagar No. 2 (Colab Laboratories, Inc., Chicago Heights, Ill) using a 1:2 dilution of 0.04 M sodium barbital-0.07 M sodium acetate buffer (pH 8.2) for the agar preparation and undiluted buffer in the electrode chambers. The method was otherwise as described by Campbell et al (8). Specific antisera were obtained from Kallestad Labs, Inc., Chaska, Minnesota. Immunoglobulin quantitation was performed by the Mancini technique (9) using plates prepared by Kallestad Labs for quantitating human IgG, IgA, and IgM at normal, low, and ultralow levels (Quanti-plate™).

Acidification tests.—After collection of two baseline urine samples, 0.1 g of ammonium chloride/kg of body weight was administered to the patient either iv or orally. Urine and serum samples were obtained thereafter at 2-hour intervals for 14 hours. Arterial blood pH was determined prior to administration of ammonium chloride and at the nadir of serum bicarbonate. All urine samples were collected under mineral oil.

Urine concentrating ability.—Urine osmolality (U_{osm}) was measured cryoscopically after overnight dehydration of the patient (16 hrs), after intramuscular (im) administration of 5 units of Pitressin tannate in oil, and after iv administration of aqueous Pitressin (300 milliunits/hr for 5 hrs). Vasopressin from the same lot used in the patient had activity verified in control patients.

RESULTS

Clinical observations.—Immediately after the initial course of isophosphamide, the patient became disoriented, lethargic, and incontinent of stool and urine for 24 hours. Blood pressure and heart rate were unchanged. Lumbar puncture revealed an opening pressure of 260 cm H_2O but was otherwise normal. Except for Day 1, when urinary incontinence supervened, urine collections were complete. Hemorrhagic cystitis occurred on Day 2 and bloody urine persisted grossly until Day 9. Microscopic hematuria continued through Day 12.

Glomerular dysfunction.—Creatinine clearance decreased to 86 ml/minute by Day 2, reached a low of 64 ml/minute on Day 4, and returned to the pretreatment level by Day 11. Serum creatinine and SUN levels remained within normal limits. Proteinuria was maximal on Day 7 (5.60 g/24 hrs). Prior to isophosphamide treatment, cellulose acetate electrophoresis and immunoelectrophoresis of the patient's urine protein (concentrated × 75) were normal. On Day 1, however, there was significant proteinuria. The electrophoretic pattern from Day 6 demonstrated the presence of all electrophoretic fractions with albumin predominating. Immunoelectrophoresis revealed a marked increase in all immunoglobulin fractions including IgM. On Day 11 the electrophoretic pattern was similar, but IgM was no longer detectable at the 75× concentration. By Day 35 (immediately before the second course of isophosphamide) the 24-hour urine protein excretion had returned to the baseline level, but the electrophoretic pattern, immunoelectrophoresis, and immunoglobulin quantitations revealed a con-

tinued low level leak of IgG and IgA. This pattern of proteinuria recurred during the second course of isophosphamide. It should be noted that hemorrhagic cystitis occurred only during the first course of therapy (a fact which complicates interpretation of urine protein excretion) but was absent during the second course when significant proteinuria was qualitatively and quantitatively similar to that of the initial course.

Tubular dysfunction.—Renal tubular cells and granular casts were observed in the urine on the day after isophosphamide treatment.

Ketonuria, glucosuria, phosphaturia, potassium loss, aminoaciduria.—Ketonuria was noted on Day 2 with negative tests for serum ketones. Glucosuria appeared on Day 2 and persisted for 5 days (fig 1), during which time serum glucose ranged from 90 to 110 mg/100 ml (simultaneous urine and serum glucose measurements). Within 24 hours after isophosphamide treatment, urine excretion of phosphorus and potassium increased markedly (fig 1) and levels remained elevated for 4 days. Serum phosphorus decreased to 1.9 mg/100 ml and serum potassium decreased to 3.5 mEq/liter. Urine calcium excretion increased progressively but still remained within the normal range. Changes in serum and urine uric acid levels in the initial treatment course are not interpretable because of the administration of allopurinol prior to isophosphamide and the patient's elevated baseline uric acid excretion. Moderate increases in urinary amino acid excretion were noted on the first day after isophosphamide treatment. On Day 2 there was marked generalized aminoaciduria which persisted through the fourth day after treatment. By Day 2 there was a tenfold or greater increase in urine excretion of lysine-ornithine, alanine, valine, and glutamic acid-citrulline, and twofold to fourfold increases in urinary arginine, leucine-isoleucine, tyrosine, phenylalanine, aspartic acid, cystine, and methionine (table 1).

Acidosis and urine acidification defect.—By Day 2 after treatment serum bicarbonate had decreased to 12 mEq/liter and serum chloride had increased to 113 mEq/liter. Serum bicar-

TABLE 1.—Urine amino acid excretion (μmols/day) after treatment with isophosphamide

Amino acid	Day								
	0*	1	2	4	35†	36	38	42	49
Lysine-ornithine	74	240	1322	851	165	268	1151	1512	231
Arginine	90	208	293	203	94	119	164	135	58
Alanine	195	868	4531	1873	400	896	3124	3159	1983
Valine	74	201	925	428	94	268	164	594	77
Leucine-isoleucine	59	136	255	252	71	119	212	243	58
Tyrosine	74	140	250	330	71	149	186	189	116
Phenylalanine	82	126	184	245	71	119	212	216	77
Aspartic acid	72	190	245	103	118	149	45	1782	847
Glutamic acid-citrulline	205	1018	25,960	315	141	5802	30,732	29,692	12,589
Cystine	90	179	236	521	71	88	141	486	154
Methionine	59	108	123	86	47	59	45	54	39

*Isophosphamide, 9.5 g.
†Isophosphamide, 6.4 g.

TABLE 2.—Urinary acidification after treatment with isophosphamide

Day	Dose of ammonium chloride	Urine pH	Serum bicarbonate (mEq/liter)	Blood pH
0*	—	5.10	26	—
2	—	6.50	12	7.39
4	—	6.60	18	7.42
6	7.0 g iv	Before: 6.91	19	7.42
		After: 6.54	14	7.39
16	7.0 g iv	Before: 6.73	23	7.51
		After: 6.54	18	7.49
27	7.2 g orally	Before: 6.11	23	7.43
		After: 5.10	14	7.37
32	—	5.92	24	—
35†	—	6.11	25	—
39	—	6.43	16	—
41	7.0 g orally	Before: 6.38	18	7.45
		After: 5.20	14	7.41
68	6.5 g iv	Before: 6.95	22	7.47
		After: 6.21	17	7.42

*Isophosphamide, 9.5 g.
†Isophosphamide, 6.4 g.

bonate did not normalize until 13 days after isophosphamide. The appearance of acidosis may have blunted the development of hypokalemia despite the hyperkaluria described above. Inappropriately alkaline urine was noted during the development of hyperchloremic acidosis (table 2). On Days 6 and 16 after isophosphamide treatment, acidification tests were performed. Despite decreases in blood pH (7.39) and serum bicarbonate (14 mEq/liter), urine acidification was significantly impaired (minimum urine pH, 6.54). On Day 27 urine acidification following administration of ammonium chloride was demonstrated, but not until serum bicarbonate decreased to 14 mEq/liter suggesting the presence of a proximal tubule acidification defect.

Urine concentrating defect.—During the first 8 days after treatment the patient lost 10.9 kg of body weight, of which 6.5 kg were explained by urinary loss. Despite frank polyuria and volume depletion, U_{osm} failed to increase above 400 mOsm/kg. On the fourth day after treatment, 5 units of Pitressin given im increased U_{osm} to only 459 mOsm/kg (table 3). Thirty-four days after isophosphamide treatment the patient continued to demonstrate impaired urinary concentrating ability in response to dehydration and exogenous vasopressin.

Re-treatment with isophosphamide.—Because of significant tumor response to isophosphamide, the patient was re-treated with a lower dose (total dose, 6.4 g) 5 weeks after the initial course. Immediately before re-treatment, the patient demonstrated normal serum electrolytes, calcium, and phosphorus levels, and had a normal urinalysis. There was no aminoaciduria, glucosuria, or ketonuria, and urinary excretion of potassium, phosphorus, uric acid, and calcium were normal. Proteinuria had decreased to 330 mg/day with a small leak of albumin, IgG, and IgA (table 4). Despite the lower retreatment dose, isophosphamide administration again resulted in glucosuria, phosphaturia, generalized aminoaciduria, kaluria, and increased

TABLE 3.—Urine concentrating ability after treatment with isophosphamide

Day	U_{osm}: overnight dehydration	U_{osm}: after im dose of Pitressin tannate
0*		
4		439
5		459‡
12	453	—
16	355	454
25	512	—
26	—	616
34	609	—
35†	—	—
41	370	398
50	389	552
70	482	602
71	—	580

*Isophosphamide, 9.5 g.
†Isophosphamide, 6.4 g.
‡After aqueous Pitressin given iv at 300 milliunits/hr for 5 hrs.

proteinuria. The abnormalities persisted for 7–9 days. During the re-treatment course, hyperuricosuria was not obscured by allopurinol administration. Creatinine clearance fell from 113 to 62 ml/minute on the day after isophosphamide, remained at this level until the fifth day after treatment, and gradually returned to normal on Day 10. During re-treatment, the patient again showed a frank defect in urinary acidification. Serum bicarbonate fell from 25 to 16 mEq/liter and serum chloride increased from 101 to 111 mEq/liter. Urinary pH was measured daily and failed to decline below 6.38. When serum bicarbonate decreased from 18 to 14 mEq/liter with administration of ammonium chloride on Day 6 of the re-treatment course, urine pH decreased to 5.20. Although serum electrolyte levels normalized by Day 9, impaired urine acidification was still present 32 days after isophosphamide re-treatment, as was impaired response to vasopressin (tables 2 and 3). No tumor response was observed after this second course of isophosphamide.

DISCUSSION

The renal tubular defects we have observed after administration of isophosphamide consisted of generalized aminoaciduria, phosphaturia, glucosuria, urine potassium loss, and defective urinary acidification and concentrating functions. The defect in urine concentrating ability may be secondary to (*a*) defective sodium reabsorption in the loop of Henle with resultant solute diuresis and dissipation of the medullary osmotic gradient, (*b*) the inability of the collecting duct to respond to vasopressin, or (*c*) a combination of these. The hyperchloremic acidosis observed immediately following isophosphamide could represent proximal or distal renal tubular acidosis or both. However, the ability to normally acidify the urine on Day 27 (pH, 5.10) after a drop in the serum bicarbonate level to 14 mEq/liter suggests a proximal acidification defect. The inability to acidify the urine on Day 2 despite a lower serum bicarbonate level (12 mEq/liter) could represent a superimposed distal acidification defect or a more severe proximal defect that had partially corrected itself by Day 27. The ketonuria observed in our patient probably reflects a defect in organic acid reabsorption sometimes seen with the adult Fanconi syndrome (10); however, the development of systemic ketosis with decreased caloric intake immediately after isophosphamide treatment cannot be excluded.

TABLE 4.—Twenty-four-hour urinary protein excretion (mg) after treatment with isophosphamide

Protein	Day 0*	6	8	11	35†	36	55
Total protein	360	3640	4300	2740	330	4940	300
Albumin	150	1090	1550	1120	140	2320	130
IgG	3	830	650	320	50	430	20
IgA	< 2	140	100	60	3	90	2
IgM	0	19	8	6	0	8	0

*Isophosphamide, 9.5 g.
†Isophosphamide, 6.4 g.

The adult Fanconi syndrome has been observed after the use of two other antineoplastic agents (streptozotocin [11], a nitrosourea, and isophthalanilide [12], a terephthalanilide derivative) but not after isophosphamide or other alkylating agents. We have prospectively studied 22 patients receiving high-dose cyclophosphamide therapy ($\geq$ 50 mg/kg of body weight) and have not observed proteinuria, evidence of urine acidification defect, or alteration in tubular handling of glucose, potassium, phosphorus, or uric acid (13). The latter observations suggest that the development of the adult Fanconi syndrome in the patient reported here is a specific effect of isophosphamide. Furthermore, no renal dysfunction was observed in our patient after previous treatment with cyclophosphamide, methotrexate, vincristine, cytosine arabinoside, and 5-fluorouracil. We have since studied two additional patients receiving isophosphamide at a reduced dosage (3.5 g/m^2). Both manifested nonselective proteinuria similar to the patient described herein, but tubular dysfunction was not noted at this lower dosage. The tubular and glomerular defects which followed both courses of isophosphamide were largely reversible by 5 weeks; there appeared to be no cumulative effects of the two courses of therapy, although our patient was left with slight impairment of urinary acidification and concentrating ability.

The acute development of generalized tubular dysfunction after the use of isophosphamide resulted in significant management problems including acidosis (serum bicarbonate as low as 12 mEq/liter) and nephrogenic diabetes insipidus (with a resultant 6.5-kg weight loss due to inadequate fluid replacement). Glucosuria and ketonuria, occurring concomitantly in such patients, may lead to the mistaken diagnosis of diabetes mellitus.

LIST OF COMPOUNDS

Allopurinol: NSC-108836; CAS reg. No. 17795-21-0; 1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ol, monosodium salt

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate

Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide

Isophthalanilide: NSC-53212; CAS reg. No. 5262-40-8; isophthalanilide, 4', 4"-di-2-imidazolin-2-yl-, dihydrochloride

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-

Streptozotocin: NSC-85998

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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BCNU (NSC-409962) in the Treatment of Advanced Hodgkin's Disease, Lymphosarcoma, and Reticulum Cell Sarcoma[1,2,3]

Vishram B. Rege[4] and Albert H. Owens, Jr.[5,6]

SUMMARY

BCNU was administered to 44 randomized patients with advanced Hodgkin's disease, lymphosarcoma, or reticulum cell sarcoma; two schedules and two dose levels for each schedule were used. Nineteen of the 44 patients (43%) showed a therapeutic response; five patients had complete regressions and 14 had regressions greater than 50%. The breakdown of responses was as follows: 16 of 33 patients (48%) with Hodgkin's disease; two of seven with lymphosarcoma; and one of four with reticulum cell sarcoma. The responses did not vary significantly by dose or schedule. The duration of response varied from 40 to 771+ days. No evidence of cross resistance with prior alkylating agents, mechlorethamine and cyclophosphamide, was noted. The toxic reactions observed most often were bone marrow depression, nausea, and vomiting. There were two deaths related to toxicity. For the various regimens the median nadirs of leukopenia ranged from 1.9 to 3.1 $\times$ 10^3 cells/mm^3, and they occurred on medians of 30–43 days after the start of treatment. The median numbers of days to recovery from the nadirs were between 11 and 16 days. The equivalent figures for platelet toxicity and recovery were 28–51 $\times$ 10^3 cells/mm^3 (median nadirs), 24–42 days (median days of nadir occurrence), and 7–22 days (median days to recovery). Results indicate a definite role for BCNU, especially in the treatment of Hodgkin's disease.

[Cancer Chemother Rep 58:383–392, 1974]

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[1] Received Sept 17, 1973; revised Dec 17, 1973; accepted Jan 8, 1974.

[2] Presented in part at the meeting of the American Society of Clinical Oncology, San Francisco, Calif, Mar 22, 1969.

[3] BCNU: CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-. Supplied by Drug Research and Development (formerly Cancer Chemotherapy National Service Center), Division of Cancer Treatment, National Cancer Institute (NCI).

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[6] This study was done by the Acute Leukemia Group B (*James F. Holland*, chairman; *Oliver Glidewell*, statistician [Operations Office, CA-10456]) and the Eastern Cooperative Oncology Group (*Bruce I. Shnider*, chairman [*Paul P. Carbone*, current chairman]). The following institutions participated in the study: Acute Leukemia Group B—McGill University Hospitals (Royal Victoria Hospital), Montreal, Canada (*Louis Lowenstein* [deceased]); Medical College of Virginia, Richmond (*John H. Moon* [deceased], CA-03735); New York Hospital, Cornell Medical Center & Affiliates, New York (*Richard T. Silver*, CA-07968); Rhode Island Hospital, Providence (*Louis A. Leone*, CA-08025); Roswell Park Memorial Institute, Buffalo, NY (*Leon Stutzman*, CA-02599); SUNY Affiliated Hospitals (Downstate), Brooklyn, NY (*Fred Rosner*, CA-12886); and West Virginia University Medical Center, Morgantown (*John B. Harley*, CA-07757). Eastern Cooperative Oncology Group—Albany Medical College, NY (*John Horton*, CA-06594); Boston University, Mass (*Albert Schilling*, CA-11109); Georgetown University School of Medicine, Washington, DC (*Bruce I. Shnider*, CA-02824); Jackson Memorial Hospital, Miami, Fla (*Jacob Colsky*, CA-02822); Johns Hopkins

BCNU has been shown to have marked antileukemic (1–6) and antitumor activities in experimental animals (7) and in clinical trials (8–10). The present randomized study was designed jointly by members of the Acute Leukemia Group B and the Eastern Cooperative Oncology Group to determine the effectiveness of BCNU in the treatment of Hodgkin's disease and lymphomas, and also to obtain toxicologic data on this drug. Two dose levels were used for each of the two schedules of administration.

MATERIALS AND METHODS

Patient Selection

All patients considered for this study had a diagnosis of Hodgkin's disease or lymphoma (including lymphocytic lymphosarcoma, lymphoblastic lymphosarcoma, and reticulum cell sarcoma, but not giant follicle lymphoma or reticulum cell sarcoma of the bone) as established by a biopsy. In addition, each patient had: (*a*) no significant lymphocytic infiltration of the bone marrow as evidenced by bone marrow biopsy or as reflected by a peripheral white blood cell count (wbc) over 15,000 cells/mm^3 with over 50% lymphocytes; (*b*) more than one area of tumor involvement and either stage III or IV disease; (*c*) tumor masses that were measurable either by calipers or on roentgenograms; (*d*) adequate bone marrow function as indicated by a total wbc of 4000 cells/mm^3 and a platelet count of over 100,000 platelets/mm^3; (*e*) adequate renal function as indicated by a blood urea nitrogen (BUN) value of 25 mg/100 ml or less or a nonprotein nitrogen (NPN) value of 40 mg/100 ml or less; (*f*) no chemotherapy, extensive irradiation, or high-dose corticosteroid therapy for 2 weeks prior to entry into the study; and (*g*) an expected survival of more than 2 months.

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Supported by Public Health Service grants (CA) from the NCI, National Institutes of Health, Department of Health, Education, and Welfare.

Mechanics of Study

Those patients who fulfilled the criteria were randomly assigned to one of the four regimens of treatment on the BCNU protocol as shown in the schema of table 1. The initial evaluation included: history, physical examination, laboratory studies (hemoglobin or hematocrit values, leukocyte [total and differential] counts, platelet count, BUN or NPN, uric acid, alkaline phosphatase, serum glutamic oxaloacetic transaminase (SGOT), total serum protein, albumin-globulin ratio, serum protein electrophoretic determinations), and chest roentgenograms. During therapy, measurements of palpable lesions, determinations of hemoglobin or hematocrit values, and leukocyte or platelet counts were done weekly; roentgenograms (for lesions observed by these studies), BUN or NPN, alkaline phosphatase, and SGOT determinations were done every 2 weeks.

BCNU was supplied as a lyophilized powder in 100-mg vials. A separate 3-ml vial of 50% alcohol was supplied as a diluent. For use, 100 mg of BCNU was dissolved in the diluent. After further dilution with 5 ml of normal saline, the compound was added to an infusion bottle of 5% glucose/water and the dose was administered intravenously (iv) over a 30-minute period. Drug modification and hematologic toxicity, as outlined in table 2, served as guidelines for reduction in the BCNU dose level.

Patients who achieved remission (complete or partial) by Day 41 of the study became eligible for maintenance treatment with BCNU; the investigator determined the dose level through analysis of the initial dose response. Those who failed to achieve remission (no change or progression) by Day 41 of the study were considered to be off-study.

Definitions of responses which included complete regression (CR), partial regression (PR), no change (NC), and progression (P) are as outlined in a previous group study (11). Patients who had a CR or PR were considered to be responders, and those who had NC or P were considered to be nonresponders. Adequate trial included treatment which produced toxicity and which, in the absence of response,

TABLE 1.—BCNU protocol schema

Randomized regimens	Induction phase	Response	Maintenance phase
I	Single high: 250 mg/m², iv single dose	CR or PR	Maintenance dose determined by the investigator based on analysis of initial dose response
II	Single low: 168 mg/m², iv single dose		Maintenance treatment until relapse, then off-study
III	Weekly high: 90 mg/m², iv weekly × 6	NC or P	Off-study
IV	Weekly low: 60 mg/m², iv weekly × 6		

TABLE 2.—Hematologic toxicity score and drug modification

Toxicity score	Peripheral blood cell counts: Wbc (cells/mm³)	Peripheral blood cell counts: Platelets (cells/mm³)	Drug dose
0 = None	≥5000	≥100,000	Full dose
1 + = Mild	<5000–4000	≥100,000	Half dose
2 + = Moderate	<4000–3500	<100,000–75,000	Half dose
3 + = Severe	<3500	< 75,000	No drug
4 + = Life-threatening or fatal	Same counts as for 3+ toxicity plus infection, bleeding, etc		No drug

was continued so that the duration of drug administration and subsequent toxicity was at least 42 days (or, in the case of the single-injection regimen, the patient was observed for that duration). Patients whose objective tumor measurements increased to at least 125% of the initial sum of the products of the diameters after 28 days of treatment and subsequent toxicity were also considered to have had an adequate trial.

RESULTS

Sixty-eight patients were entered into the study program between January 1965 and May 1967. Twenty-four patients were invalidated from the study protocol for the following major reasons: (*a*) not eligible for the protocol because of diagnostic deviation or because of lack of measurable disease (ten patients); (*b*) improper dose level, or concomitant therapy with prednisone[7] or extensive radiation (eight patients); (*c*) inadequate trial (six patients).

Forty-four patients were considered valid. The ages of these patients ranged from 19 to 78 years. There were 30 men and 14 women.

[7] Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

TABLE 3.—Response by dose and schedule

Dose regimen	Total No. of patients	No. of patients responding			
		CR	PR	NC	P
Single high	14	3	2	4	5
Single low	10	2	4	2	2
Weekly high	10	0	5	1	4
Weekly low	10	0	3	2	5
Total	44	5	14	9	16

Thirty-three patients had a diagnosis of Hodgkin's disease, seven had lymphosarcoma, and four had reticulum cell sarcoma.

There were a total of 19 objective responses, five complete and 14 partial, with an overall response rate of 43% (19 of 44) (table 3). The probability of response (CR and PR) did not vary significantly by dose or schedule. Among all the responders, however, the chance of producing a CR was significantly greater in the single-injection regimen than in the weekly injection regimen, with no regard for dose ($P = 0.039$).

As shown in table 4, the duration of response varied from 40 to 771 days. It is of interest (but not statistically significant) that the two long-term responders were in the single-injection group.

TABLE 4.—Duration of response in days

Dose regimen	Duration of response (days) for patients responding	CR + PR (%)
Single high (5 patients)	57, 74, 85, 92, 738	36
Single low (6 patients)	51, 56, 76, 148, 182, 771	60
Weekly high (5 patients)	40, 72, 81, 84, 144	50
Weekly low (3 patients)	56, 57, 59	30

The response rate was 48% for Hodgkin's disease (16 of 33 patients, four CRs) and 27% for the lymphomas (three of 11 patients, no CRs) (table 5).

TOXICITY

The various toxic reactions and their severity are recorded in table 6. The toxic reactions observed most often were bone marrow depression, nausea, and vomiting. Thirty-four patients encountered depression of at least one of the formed elements of the blood. Thirty-one patients showed leukocytic toxicity, and 24 showed platelet toxicity. In two patients the toxicity was severe and resulted in death.

For the various regimens, the median nadirs of the wbc were between 1.9 and 3.1 × 10^3 cells/mm^3, and they occurred on medians between 30 and 43 days after the start of treatment (table 7). The median numbers of days to recover from the wbc nadirs were between 11 and 16 days. There was no remark-

TABLE 5.—Distribution of diagnosis and response by treatment regimen*

Disease	Single high		Single low		Weekly high		Weekly low		Total	
	R	NR	R	NR	R	NR	R	NR	R	NR
Hodgkin's disease	5	4	5	2	3	5	3	6	16	17
Lymphosarcoma	0	3	1	2	1	0	0	0	2	5
Reticulum cell sarcoma	0	2	0	0	1	0	0	1	1	3
	5	9	6	4	5	5	3	7	19	25
Total No. of patients	14		10		10		10		44	

*R = responders; NR = nonresponders.

TABLE 6.—Severity scoring of toxic effects

Type of toxicity*	No. of patients showing indicated degree of toxicity while receiving treatment regimen—				
	Single high	Single low	Weekly high	Weekly low	Totals
Hematologic score					
0	3	1	2	4	10
1	1	1	0	1	3
2	1	1	0	0	2
3	7	7	8	5	27
4	2	0	0	0	2
Gastrointestinal score					
0	8	6	4	3	21
1	4	3	6	6	19
2	2	1	0	1	4
3	0	0	0	0	0
4	0	0	0	0	0
Other score (renal, hepatic, etc)					
0	10	9	7	9	35
1	4	1	3	1	9
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
Total No. of evaluable patients	14	10	10	10	44

*Toxicity score: 0 = none; 1 = mild; 2 = moderate; 3 = severe; 4 = life-threatening or fatal.

able difference in the depression by dose or schedule except that depression occurred somewhat less frequently among those patients in the weekly low-dose group. When it did occur, however, it appeared to be just as severe and lasted just as long as in the other regimens. The single-high-dose group had the highest frequency of failure to recover from the wbc toxicity; the two toxic deaths were in this group.

The median nadirs of the platelet count for the various regimens were between 28,000 and 51,000 platelets/mm³ and occurred on medians between 24 and 42 days after the start of treatment (table 8). The median numbers of days to recover from the platelet count nadirs were between 7 and 22 days. The maximum platelet count depression occurred later on the weekly injection schedule than on the single-injection schedule but was of nearly equal severity and probably required just as long for recovery.

Nausea and vomiting occurred in 19 patients during or a few hours after the administration of BCNU. They were more prolonged in four patients, lasting up to 24 hours, but in all patients they were controlled with antiemetics.

Only one patient showed a progressive elevation of BUN, bilirubin, and alkaline phosphatase values. This patient also had other evidence of progressive disease which might account for the azotemia and abnormal liver function. Five other patients had abnormal liver function test results (bilirubin, alkaline phosphatase, SGOT, and lactic dehydrogenase) while receiving therapy.

Transient local venous pain occurred in one patient, and in another patient there was transient burning at the injection site with a generalized itching and flushing of the face.

At the time of evaluation, five deaths had occurred either during or after drug administration. These cases were carefully reviewed, and based upon the investigator's

TABLE 7.—Hematologic (wbc) toxicity

	Single high	Single low	Weekly high	Weekly low
		Nadir of wbc (cells × 10³/mm³)		
Minimum	0.3	1.3	1.6	0.4
Median	2.2	1.9	3.1	3.1
Maximum	4.3	4.7	3.9	4.5
No. of patients with depression <5.0	10	8*	8	5
No. of patients with no depression <5.0	4	2	2	5
		Day of wbc nadir		
Earliest	20	7	33	21
Median	30	43	43	39
Latest	57	52	68	56
		No. of days to recovery from wbc nadir		
Shortest	7	3	2	7
Median	16	14	11	16
Longest	35	24	41	27
No. of patients with no recovery before going off-study	4	1	2	1

*In one patient toxicity was difficult to gauge.

TABLE 8.—Hematologic (platelet) toxicity

	Single high	Single low	Weekly high	Weekly low
		Nadir of platelet count (cells × 10³/mm³)		
Minimum	0	29	29	18
Median	28	51	46	50
Maximum	73	82	80	92
No. of patients with depression <100	9	5	6	4
No. of patients with no depression <100	5	5	4	6
		Day of platelet count nadir		
Earliest	19	21	40	32
Median	27	24	42	37
Latest	57	38	93	50
		No. of days to recovery from platelet count nadir		
Shortest	5	3	7	3
Median	18	12	22	7
Longest	31	39	43	11
No. of patients with no recovery before going off-study	3	0	0	1

opinion and the available postmortem examination reports two of the deaths were considered to be related to drug toxicity. Both of these patients had a diagnosis of reticulum cell sarcoma and were receiving treatment according to the single-high-dose regimen.

Patient No. 1, a 49-year-old woman with reticulum cell sarcoma, received a single 370-mg dose of BCNU. From a total wbc of 8100 cells/mm^3 on Day 1, the count dropped to 450 cells/mm^3 on Day 26; the patient died on Day 27 with infection. A postmortem examination was not performed, but the death was considered to be directly related to drug toxicity.

Patient No. 2, a 38-year-old man who also had reticulum cell sarcoma, received a single 400-mg dose of BCNU. This patient had evidence of bone marrow invasion by tumor prior to the start of therapy with BCNU. He was treated with corticosteroids because of his clinical deterioration even though his peripheral blood cell counts were normal. On Day 1, this patient had wbc and platelet counts of 6100 and 510,000 cells/mm^3, respectively. By Day 20, the platelet count had dropped to 30,000 platelets/mm^3, and on Day 27 it had dropped further to 0 platelets/mm^3 with a wbc of 800 cells/mm^3. The patient died on Day 28. Postmortem examination revealed (*a*) extensive malignant lymphomatous involvement of various organs including the bone marrow; (*b*) hemorrhage of the stomach due to lymphoma but complicated by glucocorticoid therapy and thrombocytopenia; and (*c*) icterus secondary to hepatomegaly with lymphomatous involvement, possibly due to BCNU therapy. In this case, death was considered to be primarily the result of progressive disease, although BCNU treatment was felt to have contributed to the fatal outcome.

Efforts were made to correlate hematologic toxicity with response (table 9): seven of 19 patients (37%) responded in the absence of platelet count depression below 100,000 platelets/mm^3 but had a median drop in platelet count of 160,000 platelets/mm^3; twelve of 23 (52%) responded who had a platelet count depression to below 100,000 platelets/mm^3 and a median drop in platelet count of 195,000 platelets/mm^3. There was no indication that wbc depression by itself was associated with an increased likelihood of response.

In respect to nearly every characteristic examined, the 19 responders and the 25 nonresponders were remarkably similar. As shown in table 10, there was no indication that they differed in age, duration of disease prior to this study, drugs received previously, response to these drugs, amount of BCNU received, or frequency of different diagnoses.

TABLE 9.—Hematologic toxicity and response

Platelet depression ($\times 10^3$ cells/mm^3)	Wbc depression ($\times 10^3$ cells/mm^3)	No. of patients*	No. of responding patients	No. of nonresponding patients	Response rate (%)
None	None	9	4	5	44
None	<5.0	10	3	7	30
<100	None	3	0	3	0
50–99	<5.0	6	3	3	50
<50	<5.0	14	9	5	64
Total		42	19	23	45

*Two cases were not evaluable for maximum depression of peripheral blood elements and have been excluded from this table. Of the 42 evaluable patients, 30 patients had wbc $<5 \times 10^3$ cells/mm^3 (15 responders, 50% response rate) and 12 patients had no wbc depression (4 responders, 33% response rate). Twenty-three of 42 patients had platelet counts $<100 \times 10^3$ cells/mm^3 (12 responders, 52% response rate) and 19 patients had no platelet count depression (7 responders, 37% response rate).

TABLE 10.—Comparability of responders and nonresponders

Characteristics	Responders (19 patients)	Nonresponders (25 patients)
Men	74%	64%
Women	26%	36%
Age (yrs):		
Youngest	21	19
Median	33	42
Oldest	78	65
Intervals (mos) from diagnosis to BCNU study:		
Shortest	4	2
Median	26	29
Longest	118	192
No previous response	32%	20%
Three or more previous responses	32%	36%
Total BCNU (mg):		
Least	245	200
Median	385	425
Maximum	1190	960
Measured tumors:		
Nodes only	33%	48%
Nodes + spleen only	39%	20%
Other	28%	32%

DISCUSSION

If usefulness of a drug in the experimental animal system (1,4,7) is any consideration, BCNU is an effective drug. In clinical trials DeVita et al (8) have shown an overall response of 21% in a wide variety of malignant conditions. Lessner (9), of the Southeastern Cancer Chemotherapy Cooperative Study Group, has reported an overall response rate of 55% (17 of 31 patients) and Young et al (10) reported 47% (21 of 45 patients) in advanced Hodgkin's disease treated with BCNU. In the present study, the overall response rate for Hodgkin's disease and the lymphomas was 43% (19 of 44 patients). The patients with Hodgkin's disease appeared to respond better (16 of 33 patients) than those with the lymphomas (three of 11 patients); however, the latter group was so small that no precise statements could be made. If one considers the fact that these patients had extensive prior treatment with chemotherapy and in some cases with radiotherapy as well, then the clinical experience with BCNU appears to be quite encouraging.

BCNU has been shown to have a high therapeutic ratio reflecting the activity over a wide range of doses (4). Also, it was effective regardless of the treatment schedule with evidence of increased survival on various schedules (4). Similar observations were made in the current study where the probability of response (CR or PR) did not vary significantly by dose or schedule.

Patients who had failed to respond to prior therapy were as likely to respond to BCNU as those patients who had demonstrated responsiveness previously (table 11). Also, the responses to each of the several agents given previously were similar for BCNU responders and nonresponders.

Toxicity

Hepatic and renal dysfunction were slight and the only prolonged and major toxic effects

were related to the hematopoietic system. Delayed toxicity (as noted by others) with a fall in the leukocyte and platelet counts in the peripheral blood was observed between 4 and 6 weeks after the start of therapy. In most cases this was reversible, with recovery in the wbc and platelet count occurring between 1 and 3 weeks after the counts had reached their nadir.

The cause of delayed toxicity is not clear, but Tsukagoshi et al (12) have shown a decrease in the activity of nicotinamide adenine dinucleotide glycohydrolase (NADase) in the various organs of mice treated with BCNU. After an initial increase there was a fall in the NADase activity of the spleen, reaching a nadir in 8–11 days after the start of therapy and followed by a slow recovery. The authors suggest a similarity between the delayed and prolonged toxic effects of BCNU observed in clinical studies by others and the delayed and prolonged suppression of NADase activity by BCNU noted by them in animal studies.

The similarity of the biologic effects of BCNU to those of the accepted alkylating agents has led to its inclusion in this class of agents (13–16). This inclusion is now questioned by many (4,17,18) on the basis of laboratory studies. In our clinical studies, the response to BCNU did not appear to be related to the response to prior alkylating agents, mechlorethamine and cyclophosphamide.[8] Somewhat similar conclusions were drawn by Lessner (9).

Of the nine patients initially sensitive (responders) to cyclophosphamide and the 18 sensitive to mechlorethamine (who then became resistant to these agents), four and six patients, respectively, showed subsequent sensitivity to BCNU. Also, of the 13 nonresponders to cyclophosphamide and the six nonresponders to mechlorethamine, five and four, respectively, showed a subsequent

TABLE 12.—Distribution of responses to BCNU and prior alkylating agents*

Treatment	R	NR
Cyclophosphamide (22 patients)	9	13
Mechlorethamine (24 patients)	18	6
Subsequent BCNU treatment		
Cyclophosphamide R (9 patients)	4	5
Cyclophosphamide NR (13 patients)	5	8
Mechlorethamine R (18 patients)	6	12
Mechlorethamine NR (6 patients)	4	2

*R = responders, CR or PR; NR = nonresponders, NC or P.

[8] Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; HN2; nitrogen mustard.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino] tetrahydro-, 2-oxide, monohydrate; Cytoxan.

TABLE 11.—Response to BCNU as related to response to previous drugs

Previous treatment	BCNU responders			BCNU nonresponders		
	No. treated (%)	No. of responses	Response rate (%)	No. treated (%)	No. of responses	Response rate (%)
Vincristine	5 (26)	4	80	8 (32)	3	38
Vinblastine*	13 (68)	8	62	15 (60)	10	67
Cyclophosphamide	9 (47)	3	33	13 (52)	6	46
Mechlorethamine	10 (53)	6	60	14 (56)	12	86
Procarbazine	3 (16)	1	33	2 (8)	1	50
Steroids	3 (16)	1	33	5 (20)	3	60
X-ray	9 (47)	8	89	11 (44)	9	82
Other drugs	2 (11)	1	50	4 (16)	2	50

* Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate.

sensitivity to BCNU. Interestingly, the quality of therapeutic response to BCNU in some cases was also superior to that of the prior alkylating agent responses. In view of all of this, one wonders if alkylation is the sole mode of action of BCNU.

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Phase I Clinical Trial of a Single-Dose and Two Weekly Schedules of ICRF-159 (NSC-129943) [1,2]

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Oleg S. Selawry,[3] and Samuel G. Taylor III [4]

SUMMARY

Initial clinical (phase I) evaluation of ICRF-159, an oral antineoplastic agent of novel structure, was carried out in 38 patients with advanced neoplastic disease using single doses and two weekly schedules. Single doses of 1.0–10.5 g/m² were given and resulted in only mild and inconsistent hematologic toxicity at all doses above 1.0 g/m². A weekly schedule of 3.0 g/m² as a single weekly dose × 6 (schedule I) produced only mild to moderate leukopenia. A dose of 3.0 g/m² given as 1.0 g/m² every 6 hours × 3 once weekly × 6 (schedule II) produced moderate to marked leukopenia. No important toxicity other than leukopenia was seen. Schedule II is recommended for phase II trial in patients who have not had extensive prior chemotherapy.

[Cancer Chemother Rep 58:393–400, 1974]

ICRF-159 is a synthetic antineoplastic agent developed by Creighton, Hellmann, and Whitecross (1) as a nonpolar derivative of the complexing agent ethylenediaminetetraacetic acid. Its structure is shown in figure 1. It produces a greater than 100% increase in lifespan against L1210 leukemia in mice on a Day 1, 5, and 9 treatment schedule, which is the optimal schedule of administration (2). It also prevents the development of pulmonary metastases in animals with subcutaneously implanted Lewis lung tumor (3). It shows a wide spectrum of antitumor activity against various animal tumors[6] and has been used clinically both alone and in combination (4,5). We report the tolerance to three dosage regimens: (*a*) single doses of from 1.0 to 10.5 g/m² of body surface area, (*b*) weekly doses starting at 3.0 g/m², and (*c*) weekly doses starting at 3.0 g/m² given in three divided doses at 6-hour intervals.

Materials and Methods

Patients

Patients with histologically confirmed cancer not amenable to conventional treatment were eligible for inclusion in the study provided their renal function was adequate as determined by a blood urea nitrogen (BUN) level of less than

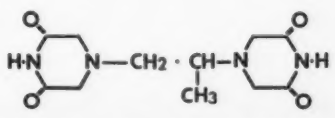

FIGURE 1.—Structure of ICRF-159.

[1] Received Sept 24, 1973; revised Dec 20, 1973; accepted Jan 14, 1974.

[2] Chemical nomenclature, NSC numbers, and CAS registry numbers for all compounds mentioned in this paper are included in the List of Compounds at the end of the text. ICRF-159 was supplied by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute (NCI).

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[6] Friedman MA, and Carter SK. Clinical brochure: ICRF 159 (NSC-129943). Prepared by the NCI, Bethesda, Md, 1970.

25 mg/100 ml and a serum creatinine level of less than 1.5 mg/100 ml. Other requirements included a serum bilirubin level of less than 1.5 mg/100 ml, a serum glutamic oxaloacetic transaminase (SGOT) level of less than 100 IU, an absence of gross edema, pretreatment white blood cell (wbc) and platelet counts in excess of 5000 and 100,000 cells/mm^3 respectively, and a sufficient interval after previous chemotherapy to avoid possible additive toxicity (2–8 weeks, depending on the preceding treatment). Patients receiving nontoxic doses of ICRF-159 were eligible for a subsequent course at the next level after an interval of at least 2 weeks. Patients who had toxic reactions were not reentered into the study with the following exceptions: patients who received single doses and had mild leukopenia (wbc $> 4.0 \times 10^3$ cells/mm^3, normal platelet count) subsequently received larger single doses after the clearing of all toxicity; all patients who received 10.5 g/m^2 as a single dose subsequently received 3.0 g/m^2 as a single dose weekly starting not less than 3 weeks after the single dose and after the clearing of all toxicity. One patient who had mild leukopenia (wbc $> 4.0 \times 10^3$ cells/mm^3, normal platelet count) and had received treatment weekly according to schedule I (*see* below) subsequently received weekly schedule II treatment, and one patient who received weekly schedule I and had no toxicity subsequently received weekly schedule II. In both cases, an interval of 3 weeks separated the two weekly schedules.

Determinations of wbc, differential leukocyte, platelet, and reticulocyte counts were performed twice weekly during treatment and for at least 3 weeks after the completion of each treatment course. Urinalysis and determinations of hematocrit, BUN, serum creatinine, SGOT, alkaline phosphatase, bilirubin, total serum proteins, serum albumin, and serum calcium values were performed once weekly. Measurable tumors were measured by caliper using the product of the greatest horizontal and vertical diameters. Additional examinations such as skeletal and chest roentgenograms were obtained before the study. The chest roentgenograms were repeated every 2 weeks whenever measurable tumors were present; for patients without measurable tumors, roentgenographic examinations were repeated at the end of each study or when indicated clinically. The patients treated, their diagnoses, and their prior treatment are shown in table 1.

Drug Administration

ICRF-159 was supplied as tablets of 50 and 250 mg. They were stored at room temperature and given orally. Patients receiving the drug according to schedule I received 3.0 g/m^2 as a single dose on Day 1. This dose was repeated weekly for 6 weeks in the absence of toxicity. In the presence of a wbc less than 5000 but greater than 3000 cells/mm^3 and/or a platelet count less than 100,000 but greater than 75,000 cells/mm^3 the dose was reduced to 2.0 g/m^2/week until both hematologic parameters had recovered. In the presence of a wbc less than 3000 cells/mm^3 or a platelet count less than 75,000 cells/mm^3 treatment was stopped until both counts rose above these levels. Patients with a wbc less than 1000 cells/mm^3 received no further ICRF-159. Patients treated according to schedule II received 3.0 g/m^2 on Day 1 divided into three equal doses given 6 hours apart. This was repeated weekly in the absence of toxicity. In the presence of hematologic toxicity the same dose reduction was used as for schedule I. Patients were followed for 3 weeks after the end of treatment. A detailed study protocol is available upon request.

RESULTS

Single-Dose Treatment

Thirty patients received 35 courses of ICRF-159 as a single dose. One patient (No. 35) had vomiting for 4 days after treatment (3.3 g/m^2) and one patient (No. 2) had very slight alopecia after 7.9 g/m^2. Apart from this, the only toxicity noted was hematologic (table 2). Toxicity was principally leukopenia, which occurred erratically at all doses greater than 1.0 g/m^2, was mild (a wbc $<$ 3000 cells/mm^3 was not recorded), and showed essentially no dose response. Thrombocytopenia occurred in only two patients and one of these (No. 23), who received 5.9 g/m^2 and had thrombocytopenia to 64,000 cells/mm^3, was a chronic alcoholic who subsequently had thrombocytopenia of 36,000 cells/mm^3 in the absence of any drug therapy.

TABLE 1.—Diagnoses and prior therapy in patients treated with ICRF-159

Patient No.	Age (yrs)	Diagnosis	Previous treatment*
1	48	Epidermoid carcinoma, lung	R, cyclophosphamide
2	62	Epidermoid carcinoma, lung	R, isophosphamide
3	68	Epidermoid carcinoma, lung	R
4	59	Epidermoid carcinoma, lung	R, mechlorethamine, methotrexate, CCNU, hydroxyurea
5	47	Adenocarcinoma, lung	R, streptozotocin, bleomycin
6	42	Adenocarcinoma, lung	Isophosphamide
7	46	Anaplastic large cell carcinoma, lung	S, R
8	34	Anaplastic large cell carcinoma, lung	R, vincristine, hydroxyurea, CCNU, procarbazine, NSC-45388
9	61	Anaplastic large cell carcinoma, lung	CCNU, NSC-141540
10	56	Anaplastic large cell carcinoma, lung	R
11	72	Adenocarcinoma, colon	S, 5-FU
12	69	Adenocarcinoma, colon	S
13	77	Adenocarcinoma, colon	5-FU
14	62	Adenocarcinoma, rectum	S, R
15	55	Adenocarcinoma, rectum	R, 5-FU, cyclophosphamide
16	47	Adenocarcinoma, stomach	S, isophosphamide
17	60	Adenocarcinoma, stomach	S, camptothecin, isophosphamide
18	55	Adenocarcinoma, stomach	5-FU
19	49	Epidermoid carcinoma, esophagus	S, R
20	60	Epidermoid carcinoma, esophagus	Streptozotocin, bleomycin
21	48	Adenocarcinoma, breast	S, R, 5-FU
22	42	Adenocarcinoma, breast	S, R, mechlorethamine, 5-FU
23	54	Epidermoid carcinoma, head and neck	Isophosphamide
24	51	Epidermoid carcinoma, head and neck	—
25	63	Adenocarcinoma, salivary gland	S, R

TABLE 1.—Diagnoses and prior therapy in patients treated with ICRF-159—Continued

Patient No.	Age (yrs)	Diagnosis	Previous treatment*
26	55	Clear cell carcinoma, kidney	R, CCNU, streptozotocin, bleomycin
27	49	Clear cell carcinoma, kidney	—
28	46	Malignant adnexal tumor, finger	S, 5-FU, bleomycin
29	78	Adenocarcinoma, pancreas	S, isophosphamide
30	51	Transitional cell carcinoma, bladder	—
31	55	Class V sputum cytology	—
32	52	Anaplastic carcinoma, primary site unknown	S, R, camptothecin, bleomycin, CCNU, methotrexate
33	83	Anaplastic carcinoma, primary site unknown	R, NSC-122819, camptothecin
34	49	Anaplastic carcinoma, primary site unknown	NSC-141540
35	36	Synovial sarcoma, knee	S, cyclophosphamide, 5-FU, bleomycin, methotrexate
36	57	Fibrosarcoma, retroperitoneum	S, isophosphamide, adriamycin, cyclophosphamide, methotrexate, actinomycin D
37	48	Fibrosarcoma, back	Adriamycin
38	54	Fibrosarcoma, back	S

*S = surgery; R = radiotherapy.

Since there was no dose response, all patients receiving doses greater than or equal to 2.0 g/m² have been pooled for the following analysis. The mean overall decrease in wbc was 32%. There was virtually no differential effect on granulocytes (73% pretreatment, 68% at the nadir of wbc). The decrease in platelets was 22%. The median time to nadir was 12 days in patients with leukopenia. The reticulocyte count decreased from a mean of 1.4% to a mean of 0.6%. Median nadir of reticulocyte count was 5 days (range, 4–8 days). A fall of more than 5 percentage points in the hematocrit value was seen in four patients. No change was seen in BUN, serum creatinine, serum bilirubin, SGOT, serum alkaline phosphatase, or serum calcium levels.

Weekly Dosage Treatment

Six patients received ICRF-159 weekly on schedule I and ten on schedule II. Leukopenia and thrombocytopenia were the only drug toxic effects seen. All patients except one (No. 6) had toxic reactions to the weekly dosage. This patient did not have toxic reactions to either weekly treatment schedule. Toxicity was milder, took longer to develop, and required more drug to become apparent on schedule I than on schedule II (table 3, fig 2). Two patients receiving schedule II (Nos. 4 and 8) had pronounced

TABLE 2.—Hematologic toxicity after single doses of ICRF-159

Dose (g/m^2)	No. of patients treated	Wbc count			Platelet count		
		No. of patients with leukopenia	Nadir ($\times 10^3$ cells/mm^3)	No. of days to nadir (mean)	No. of patients with thrombocytopenia	Nadir ($\times 10^3$ cells/mm^3)	No. of days to nadir
1.0	3	0	—	—	0	—	—
2.0	2	1	4.3	12	0	—	—
3.3	5	3	4.4 (3.8–4.8)	12	0	—	—
4.4	3	2	3.2, 4.3	5, 11	0	—	—
5.9	5	3	4.6 (4.3–4.8)	12 (11–13)	1	64	13
7.9	8	5	4.1 (3.3–4.9)	15 (11–18)	1	97	11
10.5	9	3	3.6 (3.3–3.9)	13 (11–15)	0	—	—

toxic reactions (wbc 700 cells/mm^3 and platelet count 64,000 cells/mm^3 in patient No. 8; wbc 800 cells/mm^3 and platelet count 21,000 cells/mm^3 in patient No. 4). Both patients had had extensive prior treatment (table 1). Counts returned promptly to normal on cessation of treatment and were readily controlled by adjustment of dosage (fig 2). No drug-related deaths occurred. No objective tumor responses were seen; however, most patients had advanced disease which was not measurable and many had had extensive prior treatment.

DISCUSSION

ICRF-159 is an antineoplastic agent of novel structure. It is active in early mitosis (6) and inhibits DNA synthesis (7) by a mechanism which is so far unknown, though it has been suggested on the basis of in vitro studies that it might acylate biological nucleophiles (7). It has shown activity in patients with acute leukemia (8) but has shown little activity so far as a single agent against low growth fraction tumors. However, in experimental systems it has demonstrated the interesting and potentially important effect of protecting against the cardiac toxicity of the anthracycline antibiotics (9). Its use in man has so far been largely on a divided daily dose schedule [7] and since it appears from a number of aspects to be a drug worthy of further study both alone and in combination we chose to define its toxicity in weekly doses. Single-dose toxicity was evaluated before weekly doses were administered, to define more precisely the kinetics of toxicity and its quantitative relationship to dosage. From the reports of British data (10) the minimum toxic dose in man appeared to be in the order of 3.0 g/m^2, and therefore we chose

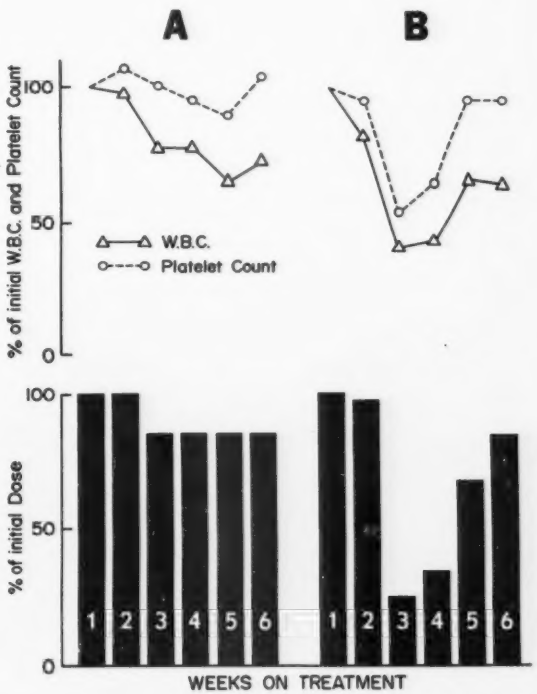

FIGURE 2.—Wbc and platelet counts in patients receiving ICRF-159 weekly on schedule I (*A*) and schedule II (*B*).

[7] *See* footnote 6.

TABLE 3.—Hematologic toxicity after weekly doses of ICRF-159

Schedule	No. of patients treated	No. of patients with wbc count—(cells/mm³)				Wks to wbc toxicity*		Wbc nadir (× 10³/mm³)		No. of patients with platelet count—(cells/mm³)		
		> 5000	3000–4900	1000–2900	< 1000	Median	Range	Median	Range	> 100,000	99,000–75,000	< 74,000
I	6	1	4	1	0	3	2–6	3.8	2.7–4.9	6	0	0
II	10	1	1	6	2	2	1–2	2.1	0.7–3.9	7	1	2

*The patient who did not have hematologic toxicity is not included in these figures.

one third of this dose as a starting dose and escalated it as indicated in table 4. Dose-limiting toxicity, however, was not seen at any dose up to 10.5 g/m² nor did there seem to be a trend in the direction of more marked or more consistent toxicity. The single-dose trial was therefore terminated after the nature and kinetics of toxicity had been determined. From the results of the single-dose trial it appeared that intestinal absorption might be limited at doses above 3.0 g/m². We therefore investigated a single and a divided weekly dose starting at about one third of the highest dose given as a single dose. It did not prove necessary to escalate the dose. Dose-limiting toxicity consisting of leukopenia was achieved in nine of ten patients treated with schedule II. Marked thrombocytopenia in the absence of dose-limiting leukopenia did not occur and no other toxicity was noted. The schedule proved unacceptably toxic in only two patients who had received extensive prior treatment. The toxicity was easily manageable. Schedule I produced toxicity which was neither as consistent nor as profound as the toxicity of schedule II. No absorption studies in animals were available when the present trial was initiated. However, we have reported (11) that at doses greater than 3.0 g/m² in man the total absorption of drug is essentially independent of the dose level, and absorption is more than 100% greater on schedule II than on schedule I, confirming the impression reached clinically that poor absorption may account at least in part for the schedule dependence.

While the present report was in preparation, the results of a study on ICRF-159 given in divided doses every 8 hours for 3 days were reported (12). The maximum tolerated dose was 1.0 g/m²/day. This is in good agreement with the concepts of marked schedule dependence noted here.

From the present studies we conclude that ICRF-159 may safely be given in phase II initial therapeutic trials using schedule II (3.0 g/m² in three divided doses [6 hours apart] once weekly) except in patients who have had extensive prior chemotherapy. In these patients, an initial trial of schedule I might be used to test susceptibility to the drug. From these results it would appear that a split schedule consisting of more than three divided doses might well prove unacceptably hazardous in some patients.

Finally, it must be clear from these data that clinical trials using an orally administered drug without adequate data on gastrointestinal absorption, even when the drug has been extensively used clinically in small individual doses, can be time consuming and costly.

LIST OF COMPOUNDS

Adriamycin: NSC-123127
Actinomycin D: NSC-3053; dactinomycin
Bleomycin: NSC-125066
Camptothecin: NSC-100880; CAS reg. No. 25387-67-1
CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU
Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1
Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide
Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; HN2; nitrogen mustard
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-
NSC-45388: CAS reg. No. 4342-03-4; 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; DTIC
NSC-122819: 4'-demethylepipodophyllotoxin 9-(4,6-*O*-2-thenylidene-β-D-glucopyranoside); VM 26
NSC-141540: 4'-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside); VP-16-213
Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride
Streptozotocin: NSC-85998
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Total Body Radiation Therapy for Disseminated Lymphosarcoma: Results of a Pilot Study [1,2]

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SUMMARY

Nine patients with disseminated lymphosarcoma were treated with total body radiation (TBR) using a fractionated treatment regimen. Seven of these patients (six previously untreated) with generalized nodal disease and bone marrow infiltration received TBR alone. The other two patients (one previously untreated) had generalized nodal disease and were treated with TBR followed by total nodal radiation. The radiation therapy was uniformly well tolerated with no serious gastrointestinal or hematologic toxic effects. Three of the patients had complete remission (2, 7, and 37+ months in duration), four patients had partial regression of adenopathy with control of the disease process (1, 4, 5, and 11 months in duration), and two patients had no objective response. The eight patients with persistent or recurrent disease were treated with chemotherapy but tolerated treatment poorly, requiring delays in therapy, decreases in dosage, and/or premature discontinuation of therapy due to inadequate bone marrow reserves. Although TBR was well tolerated and produced objective disease response in seven of nine patients treated, the duration of response was relatively short and the ability to subsequently deliver effective combination chemotherapy was seriously compromised.

[Cancer Chemother Rep 58:401–405, 1974]

Total body radiation therapy (TBR) has been used for the treatment of a variety of neoplastic disorders for over 40 years (1–10).[7] The most encouraging results have been observed in patients with chronic lymphatic leukemia (6) and disseminated lymphosarcoma (4,5,7–10).

In view of the favorable preliminary experience with TBR in the management of advanced lymphosarcoma reported by others (4,5,7), a pilot study was initiated at the Baltimore Cancer Research Center, Division of Cancer Treatment, National Cancer Institute, in 1970 to further investigate the value of this therapeutic modality in patients with dissemin-

[1] Received Nov 16, 1973; revised Feb 14, 1974; accepted Feb 25, 1974.

[2] Compounds used in this study were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

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[6] The authors wish to thank Dr. Arthur Serpick for his assistance in the management of these patients, Dr. Peter Wiernik for reviewing the manuscript, and Ms. Delores Norris for secretarial assistance.

[7] Andrews GS, Sittersun BW, White DA, et al. Summary of clinical total body irradiation program. Research report from the Oak Ridge Institute of Nuclear Studies, Medical Division, 1962.

ated lymphosarcoma. This report summarizes our observations on nine patients.

PATIENT SELECTION AND METHODS

Nine patients with generalized lymphosarcoma were entered into the study. Two of the nine patients had previously received both radiotherapy and chemotherapy, while seven patients had received no prior therapy. The original diagnostic lymph node biopsies were reviewed and reclassified according to the scheme devised by Gall and Rappaport (11) for the histologic classification of non-Hodgkin's lymphomas. Seven patients had documented bone marrow involvement by malignant lymphoma in addition to generalized adenopathy, whereas two patients had generalized adenopathy without evidence of extranodal involvement (table 1). No patient had evidence of visceral involvement prior to the initiation of TBR.

TBR was administered by use of a conventional cobalt 60 device (Theratron, 80 unit). The patients were treated in a sitting position with half of each dose administered from the right and half from the left, except when doses of 5 rads or less were given. The patients were placed with the midplane at a distance of 320 cm from the source, and a dose rate of approximately 10 rads/minute was used. At the start of the study an attempt was made to deliver a single weekly dose of 25 rads to the midplane, but it was discovered that excessive depression of the white blood cell (wbc) and platelet counts occurred. Therefore, the dose rate was reduced to 15 rads/week until the platelet count fell to approximately 100,000 platelets/mm^3, at which time the dose rate was further reduced until the platelet count remained depressed below 100,000 platelets/mm^3 and therapy was then discontinued.

TBR was discontinued in one patient (No. 8) at a dose of 95 rads since it was decided to administer total nodal radiation after the TBR. This patient and patient No. 9, both of whom had disease apparently limited to the lymph nodes, received 2000 rads to standard upper mantle and inverted Y fields following TBR.

RESULTS

The TBR was tolerated without significant immediate morbidity by all patients. Vomiting, diarrhea, skin changes, and alopecia were not observed. Moderate depression in the peripheral blood counts was noted in eight of the patients (table 2); however, no patient required transfusion therapy, and bleeding or infec-

TABLE 1.—Patients with disseminated lymphosarcoma treated with TBR

Patient No.	Age, sex	Previous therapy	Histology*	Disease stage	Extranodal disease
1	58,F	None	Mixed, nodular	IV	Marrow
2	48,M	Chemotherapy, radiotherapy	PDL, nodular	IV	Marrow
3	67,M	None	WDL, nodular	IV	Marrow
4	36,M	None†	PDL, diffuse	IV	Marrow
5	40,F	None	PDL, nodular	IV	Marrow
6	41,M	None	PDL, nodular	IV	Marrow
7	50,M	None	PDL, nodular	IV	Marrow
8	47,M	None	PDL, nodular	III	—
9	46,M	Chemotherapy, radiotherapy	Mixed, nodular	III	—

*PDL = poorly differentiated lymphocytic lymphoma. WDL = well-differentiated lymphocytic lymphoma.

†Received mediastinal radiation for superior vena cava syndrome before beginning TBR.

tious complications related to blood count depression did not occur.

Response to therapy is outlined in table 3. Three patients had complete regression of all detectable disease. Four additional patients experienced definite reduction of lymph node bulk, and one of these patients (No. 3) also had no evidence of lymphoma on posttreatment bone marrow biopsy. There was no demonstrable effect of TBR in patient No. 2, whereas patient No. 4 had leukemic conversion of his lymphoma while receiving TBR.

Eight of the nine patients have required further therapy for either persistent or recurrent disease. Significant delays in initiating therapy because of persistent cytopenia occurred in two patients. Six patients were treated with combination chemotherapy (cyclophosphamide, vincristine, and prednisone in four patients; mechlorethamine, vincristine, pro-

TABLE 2.—Dosage schedules and hematologic toxicity in patients receiving TBR

Patient No.	Total dose (rads)	Weeks of treatment	Nadirs (cells/mm³)	
			Wbc	Platelets
1	150	13	2300	71,000
2	100	25	2600	69,000
3	165	14	4000	58,000
4	85	3	13,000*	20,000
5	120	14	2700	66,000
6	196	19	3400	75,000
7	160	11	3900	32,000
8	95	7	4000	108,000
9	106	3	2700	54,000

*Leukemic conversion developed during TBR.

TABLE 3.—Disease response in patients treated with TBR

Patient No.	Response	Duration of response (mos)	Site of relapse	Chemotherapy tolerance	Current status
1	Regression of nodes	11	Abdominal nodes	Poor	Alive with disease
2	Failure	—	—	Poor	Deceased
3	Regression of nodes; normal marrow	4	Axillary, supraclavicular nodes	Poor	Deceased
4	Failure	—	—	Poor	Deceased
5	Complete remission	7	Cervical, inguinal nodes; liver (?)	Poor	Alive with disease
6	Complete remission	2	Inguinal nodes; marrow	Poor	Alive in remission
7	Regression of nodes	5	Generalized adenopathy; marrow	Poor	Deceased
8*	Complete remission	37+	None	—	Alive in remission
9*	Regression of nodes	1	Leptomeninges	Poor	Deceased

*Received total nodal radiation after TBR.

carbazine, and prednisone in two patients).[8] Severe bone marrow depression (primarily thrombocytopenia) occurred in each patient despite major drug dosage reductions in five of the patients. The remaining two patients received therapy with less myelosuppressive drugs (vincristine and prednisone) due to persistent marrow depression following TBR but neither had a response. Of the seven patients receiving chemotherapy subsequent to TBR, only one (No. 6) achieved complete remission. The remaining patients have either died or have required continuous therapy in reduced dosages to control disease activity.

Of the nine patients originally treated, five have died at 3, 17, 32, 36, and 44 months following the completion of radiation therapy. The remaining four patients have survived 31, 35, 36, and 38 months.

DISCUSSION

TBR has been demonstrated to be well tolerated by patients with advanced lymphomas if a fractionated dosage schedule utilizing small, intermittent doses of radiation is employed (4,5,7–10). Our experience with this small series of patients using a fractionated dosage scheme is in agreement with previous reports. No immediate side effects of TBR were observed and hematologic toxicity was acceptable. Thrombocytopenia was the major factor limiting further radiation therapy. It has been noted (4) that patients previously treated with chemotherapy experience greater hematologic toxicity from TBR; however, we did not observe undue marrow suppression in the two previously treated patients in this study.

The clinical status of patients with advanced lymphomas and the histopathology of their tumors appear to be important prognostic indicators of response to TBR. Johnson et al (8) observed poor responses and short survival in a group of patients having visceral involvement with lymphosarcoma who were treated with TBR. Marrow infiltration with tumor did not carry the same poor prognosis, and several patients with stage IV disease on the basis of bone marrow involvement alone had control of their disease with TBR. The patients reported to derive benefit from TBR in that report all had lymphocytic lymphomas. We are not aware of published reports documenting the efficacy of TBR in patients with mixed or histiocytic lymphomas.

None of our patients had evidence of visceral involvement prior to TBR, but two patients were found to have mixed lymphomas on careful review of the diagnostic lymph node biopsies. Although both of these patients experienced regression of lymph nodes after TBR, one (No. 9) relapsed 1 month after the completion of TBR and died 2 months later. The other patient (No. 1) experienced an 11-month partial remission and is currently alive 36 months following TBR, although she has required chemotherapy and has evidence of persistent bone marrow involvement.

The potential for prolonged control of disseminated lymphoma by the use of aggressive radiation therapy is illustrated by one of our patients (No. 8) with stage III disease. To date, he has been disease-free 37 months after the completion of TBR. Like several of the patients reported by Johnson et al (7–9), he received total nodal radiation following TBR. Thus, the favorable clinical response obviously cannot be ascribed solely to TBR. The existence of long-term remissions in those patients treated with TBR plus total nodal radiation suggests that radiation may still play an important role in the treatment of disseminated lymphocytic lymphoma. It would seem important to delineate the factors which make such long-term remissions possible, although this is not possible with our single case. Since patients with visceral involvement have been shown to respond poorly to TBR and would be expected to have poor tolerance to chemotherapy in the event of relapse on the basis of our experience,

[8] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy-.

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard.

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride.

we feel that careful exclusion of hepatic and gastrointestinal involvement by lymphoma is mandatory before attempting this aggressive radiotherapy program.

In the small series of patients treated with TBR at our institution, the duration of disease control with one exception has been relatively brief. It is possible that differences in the dosage scheduling of TBR as well as a somewhat lower total radiation dose compared to Johnson's regimen may account for the lower complete remission rates and shorter remission durations observed in the present series. Although in retrospect our patients may have been able to tolerate higher doses of TBR, it was not considered advisable to continue radiation therapy in the face of persistent thrombocytopenia or leukopenia or both because of the potential risk of severe bone marrow aplasia. We elected to treat our patients primarily with chemotherapy at the time of relapse or progression of the lymphomatous process, and we experienced considerable difficulty in delivering effective therapy because of poor bone marrow tolerance. Johnson (9), however, has reported more favorable remission durations in a selected population of patients with advanced lymphosarcoma treated primarily with radiotherapy, and he states that further radiation therapy (TBR or local radiation) can be effectively administered to achieve disease control without excessive morbidity at the time of relapse.

Several recent reports (12–14) have documented the value of systemic combination chemotherapy in providing prolonged disease-free survival in many patients with disseminated non-Hodgkin's lymphomas. Furthermore, chemotherapeutic regimens can potentially be used to advantage regardless of the cell type or visceral involvement in these patients.

Assessment of the relative value of TBR as opposed to combination chemotherapy as the primary treatment modality in patients with disseminated lymphosarcoma would require a prospectively randomized clinical trial comparing TBR administered in the most effective schedule versus a combination chemotherapy regimen such as outlined by Bagley et al (14). In the meantime, however, we would caution that severe myelosuppression may result from the combined or sequential use of these two treatment modalities.

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Toxicity Study of BCNU (NSC-409962) Given Orally[1,2,3]

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Writing Committee for the Southeastern Cancer Chemotherapy Cooperative Study Group

SUMMARY

The toxicity and therapeutic efficacy of BCNU administered orally were evaluated in 104 patients with miscellaneous types of malignant diseases. Three dose schedules were employed: (*a*) a single daily dose of 20, 40, or 50 mg/m² given for 5 days; (*b*) a divided dose of 5, 10, or 12.5 mg/m² given every 6 hours for 5 days; (*c*) a single daily dose of 5, 10, or 12.5 mg/m² given for 60 days. Hematologic toxicity was mild, occurring in 21% of the patients. The limiting factor proved to be nausea and vomiting which occurred in 39% of the patients. Objective tumor regressions were noted in five of 31 patients with previously treated Hodgkin's disease.

[Cancer Chemother Rep 58:407–411, 1974]

In 1968 the Southeastern Cancer Chemotherapy Cooperative Study Group reported on the intravenous (iv) use of BCNU in a variety of human malignancies (1). Our findings at that time indicated a 55% response rate in advanced Hodgkin's disease treated with this agent alone. Subsequent papers have since confirmed these findings (2,3).

Several problems became apparent with this method of drug administration, including those of delayed hematologic toxicity, nausea, vomiting, and frequently distressing venous ir-

[1] Received Nov 29, 1973; accepted Feb 21, 1974.

[2] BCNU: CAS reg. No. 154–93–8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, National Cancer Institute (NCI).

[3] This investigation was supported by Public Health Service research grants (from the NCI, National Institutes of Health, Department of Health, Education, and Welfare) to the following institutions: CA-03177, Duke University Medical Center, Durham, NC; CA-05634, Veterans' Administration Hospital, Durham, NC; CA-03227 and CA-11263, Emory University School of Medicine, Atlanta, Ga; CA-06807, Medical College of Georgia, Augusta; CA-03337, University of Kansas Medical Center, Kansas City; CA-05641, University of Miami School of Medicine, Fla; CA-12639, Presbyterian-University of Pennsylvania Medical Center, Philadelphia; CA-07961, Temple University School of Medicine, Philadelphia, Pa; CA-03376, Washington University School of Medicine, St. Louis, Mo; and CA-12223, University of Puerto Rico School of Medicine, San Juan.

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ritation at the site of administration of the BCNU, at times necessitating discontinuation of treatment. In addition, stable remissions were difficult to maintain in some patients receiving iv treatment since hematologic toxicity would at times extend beyond the point of recurrent symptomatology. In an attempt to obviate some of these problems the Southeastern Cancer Study Group subsequently embarked on a phase I study of BCNU given orally.

DRUG CHARACTERISTICS

Utilizing ^{14}C-labeled BCNU, DeVita et al (4) have demonstrated that the drug is stable at acid pH and is rapidly absorbed from the stomach, attaining serum levels commensurate with a similar dose given iv. Fifty percent of the ^{14}C tag is excreted in the urine in 24 hours, similar to iv administration. Furthermore, similar hematopoietic toxicity was noted in monkeys and dogs when BCNU was given either orally or parenterally. Thus, it appeared that both oral and iv treatments with BCNU were quite similar in plasma levels attained, rate of plasma clearance, and toxicity. In addition, Rall, Ben, and McCarthy (5) have demonstrated antitumor effects with BCNU given orally to five patients with meningeal leukemia.

METHOD OF STUDY

Three different dosage schedules were explored as shown in table 1. Two of these, schedules *A* and *B*, consisted of 5-day courses, and the third schedule (*C*) consisted of a 60-day course. In schedule *A* the daily dose was given in 1 aliquot, whereas in schedule *B* the daily dose was divided into equal aliquots given every 6 hours for four doses. In schedule *C* the dose was given once daily for 60 days. BCNU was supplied in 2.5-, 5.0-, and 25-mg capsules which were kept in the freezer with added desiccant until used.

TABLE 1.—Treatment schedules using BCNU given orally

Schedule	Dose regimen
A: single daily dose	20 mg/m²/day × 5 days
	40 mg/m²/day × 5 days
	50 mg/m²/day × 5 days
B: divided dose	5 mg/m² every 6 hrs × 5 days
	10 mg/m² every 6 hrs × 5 days
	12.5 mg/m² every 6 hrs × 5 days
C: single daily dose	5 mg/m²/day × 60 days
	10 mg/m²/day × 60 days
	12.5 mg/m²/day × 60 days

After each course of therapy no further treatment was given for 28 days or until toxicity abated if still evident at that time. Escalation of dosage to higher levels was permitted if no toxicity had been noted at the original dose level. It became apparent early in the study that the first dosage level of all three schedules was tolerable and patients were subsequently started at the second dosage level. A minimum of six patients were entered at each dose level before the dose was increased.

Patients with metastatic neoplasms refractory to other modes of treatment who had normal blood counts and who had not received extensive prior chemotherapy or radiation therapy were eligible for this study. Pretreatment studies included a complete physical examination with measurement of any palpable tumors, adenopathy, or organomegaly, and appropriate roentgenograms. Laboratory studies included a complete blood cell count, bone marrow aspiration and biopsy, urinalysis, alkaline phosphatase, serum glutamic oxaloacetic transaminase, serum protein electrophoresis, serum uric acid, and blood urea nitrogen. Renal and hepatic function tests were obtained at least every 2 weeks.

CRITERIA FOR RESPONSE

Each study was evaluated by each principal investigator and project chairman and any discrepancies in opinion as to the therapeutic effect or toxicity were resolved by personal communication. Responses were rated as follows:

1. An "excellent" response indicated complete remission or disappearance of all objective and subjective evidence of disease.
2. A "good" response and a "fair" response indicated a 50% and 25% improvement respectively in objective evidence of disease.

CRITERIA FOR TOXICITY

Hematologic toxicity was rated as follows:

Hemoglobin toxicity: grade 1 = a 3–4.9 g/100 ml fall compared to the initial value; grade 2 = a 5 or more g/100 ml fall compared to the initial value.

Granulocyte toxicity: grade 1 = a fall below 1500 granulocytes/mm^3; grade 2 = a fall below 750 granulocytes/mm^3; grade 3 = a fall below 200 granulocytes/mm^3 associated with a life-threatening infection.

Platelet toxicity: grade 1 = a fall below 100,000 platelets/mm^3; grade 2 = a fall below 50,000 platelets/mm^3; grade 3 = a fall below 10,000 platelets/mm^3.

Hepatic and renal function toxicities were judged by the appearance of abnormal values thought to be related to drugs which returned to pretreatment levels after cessation of treatment.

RESULTS

A total of 116 studies were conducted in 91 patients of which 104 studies were evaluable as to toxicity and response. The total experience and therapeutic responses in the various tumors are presented in table 2.

Schedule A.—A total of 15 patients treated with schedule *A* were evaluable. As shown in table 3, hematologic toxicity was minimal, but gastrointestinal toxicity consisting of nausea and vomiting occurred in ten patients, three of five at the lowest dose. Therefore, this schedule was abandoned. No therapeutic responses were noted.

Schedule B.—As shown in table 3, hematologic toxicity was infrequent, occurring in nine of 34 evaluable patients, and did not seem to be dose related. Only one patient experienced granulocyte toxicity. Of the eight patients who had thrombocytopenia, five were rated as grade 1 and three were rated as grade 2 toxicities. The nadir for granulocytes was Day 20 (range, 10–25) and the nadir for platelets was Day 20 (range, 13–34). Recovery took 20 days for platelets and 17 days for granulocytes. Nausea and vomiting occurred in one half of the patients at the lower doses and in one third of the patients at the lowest dose. Only one patient with Hodgkin's disease showed a fair response.

Schedule C.—A total of 55 evaluable patients were entered on this schedule as shown in table 3. Of 17 patients receiving a single dose of 5 mg/m^2/day, three experienced nausea and

TABLE 2.—Breakdown of patients treated orally with BCNU

Neoplasm	No. of patients— Entered	Evaluable	Showing >25% improvement
Lung	10	8	
Breast	3	3	
Stomach	7	7	2*
Colon	7	6	
Cervix, vagina	5	5	
Kidney	4	3	
Head and neck	2	1	
Choriocarcinoma	2	1	
Unknown primary	13	13	
Sarcoma	6	4	
Melanoma	16	16	2*
Hodgkin's disease	32	31	5
Lymphocytic lymphoma	4	4	
Histiocytic lymphoma	2	0	
Chronic lymphocytic leukemia	3	2	
Totals	116	104	9

*Same patient who responded to each of 2 different studies.

vomiting and two had significant hematologic toxicity. Ten of 34 patients treated at doses of 10 mg/m²/day experienced nausea and vomiting, hematologic toxicity was observed in 18 instances, and anemia developed in four patients. Granulocyte toxicity occurred in six patients: three had grade 1, two had grade 2, and one had grade 3 toxicity. Thrombocytopenia occurred in seven patients: four had grade 1, three had grade 2, and two had grade 3 toxicity. The nadir for granulocytes was Day 42 (range, 31–56) and the duration averaged 28 days (range, 16–39). The nadir for platelets was Day 46 (range, 40–60) and the duration averaged 31 days (range, 21–40).

No renal toxicity was observed. One patient with Hodgkin's disease receiving schedule A had jaundice and an elevated alkaline phosphatase level. Liver biopsy was negative and it is uncertain that these findings were due to BCNU. A second patient receiving schedule B had abnormal liver function tests thought to be due to the drug.

One patient had gynecomastia while receiving schedule *B* which regressed with cessation of therapy.

The therapeutic responses to BCNU given orally were limited. As shown in table 2, only nine patients experienced a fair or better response and two of these were in the same patient. One patient with carcinoma of the stomach responded with a fair response on each of two *C* schedules. A patient with metastatic melanoma had an excellent response to the *B* schedule, and when treated again at the time of relapse he had an excellent response to schedule *C*. The remainder of the responses occurred in patients with Hodgkin's disease receiving schedule *C* where of 54 patients four had a fair, one had a good, and one had an excellent response to therapy. In our previous report 29 of 60 patients obtained this degree of response with BCNU given iv (1). The difference in response rates between oral and iv treatment with BCNU was significant ($P<0.05$).

DISCUSSION

Nausea and vomiting appeared to be the limiting factor in the oral administration of BCNU, particularly when the drug was given over 5 days. Single, low-dose, daily therapy was better tolerated, although 25% of the patients had gastrointestinal toxicity. The failure

TABLE 3.—Toxicity from BCNU given orally

Schedule	Dose (mg/m²)	No. of patients— evaluable	with nausea and vomiting	with a fall in hemoglobin level >3 g/100 ml	with nadirs— granulocytes/mm³ <1500	<750	<200	platelets/mm³ <10⁵	<5 × 10⁴	<10⁴
A: daily × 5	20	5	3	1	1	0	0	2	0	0
	40	8	5	0	0	0	0	0	0	0
	50	2	2	0	0	0	0	0	0	0
	Totals	15	10	1	1	0	0	2	0	0
B: every 6 hrs × 5	5	9	3	0	0	0	0	3	0	0
	10	17	9	0	1	0	0	1	2	0
	12.5	8	4	0	0	0	0	1	1	0
	Totals	34	16	0	1	0	0	5	3	0
C: daily × 60	5	17	3	0	1	0	0	1	0	0
	10	34	10	4	3	2	1	4	3	2
	12.5	4	1	0	0	0	0	0	1	0
	Totals	55	14	4	4	2	1	5	4	2

to administer adequate doses of the drug is reflected in the lack of myelosuppression and therapeutic responses. In contrast to our previous study (1), only 19% of the patients given BCNU orally had myelosuppression, whereas 44% of patients given BCNU iv showed significant myelotoxicity. Of the 31 evaluable patients with Hodgkin's disease entered in this study, only ten demonstrated myelosuppression and five showed objective responses. It is clear that gastrointestinal intolerance to BCNU given orally is a serious detriment to its use in the clinical management of cancer.

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Physical Activity: A Possible Determinant of Vincristine (NSC-67574) Neuropathy [1,2]

Arthur Sakamoto [3]

Brief Reports and Preliminary Communications

Vincristine neuropathy usually limits the total dose that can be administered to any patient receiving this useful chemotherapeutic agent (1,2). Freund and Kendel (3) and Daun and Hartwich (4) feel that a specific total dose of vincristine always produces functional neuronal damage. A review of the records of about 20 patients who received Frenster's low-dose combination chemotherapeutic regimen (5) indicates that the onset of vincristine neuropathy may be a function of not only the total dose administered but may also be related to the physical activity of patients receiving vincristine.

MATERIALS AND METHODS

The records of 24 patients who received Frenster's low-dose combination chemotherapeutic regimen (5) were reviewed and 20 cases were found suitable for inclusion in this study. The physical activity of each patient was categorized as follows:

0: bedridden.

I: either up in chair or in bed during most of the day; limited walking (to bathroom only or a few steps more per day).

II: activity limited to
- (*a*) dressing self daily and a little walking, or
- (*b*) doing light housework, or
- (*c*) walking one to two blocks daily.

III: activity limited to either working daily or taking complete care of his/her home.

IV: activities included vigorous work and a social life.

The onset of neuropathy was noted either by paresthesia, loss of patellar reflex, or muscle weakness. In this small series, ileus, hoarseness, and other unusual forms of vincristine neuropathy were not observed. The data of four of the 24 patients in this series were eliminated due to inadequate records or incomplete follow-up (three of the four patients received chemotherapy under the supervision of another physician).

According to the protocol as described by Frenster (5), the doses were: vincristine, 0.015 mg/kg/wk given intravenously (iv); 5-fluorouracil (5-FU),[4] 10 mg/kg/wk given iv; and cyclophosphamide,[5] 1 mg/kg/day given orally. Generally, those patients who had not received prior chemotherapy or irradiation treatment tolerated the bone marrow-suppressive effects of 5-FU and cyclophosphamide without requiring reduction of the dose level of either of the two agents. A few who had received such treatment required dose-level reduction. The dose level of vincristine was held constant until symptoms of neuropathy (either sensory [numbness and tingling of fingers and toes] or motor [absence of patellar reflex]) appeared.

Thyroid function was not routinely assessed in all patients; however, serum thyroxine (T_4) levels were available in a few.

RESULTS

In group I, two of five patients noted paresthesia as the initial manifestation of neuropathy, and both of these patients were eventually hospitalized for disabling muscle weakness. In group IV, three of six patients also noted paresthesia as the first manifestation, but none needed hospitalization for severe muscle weakness.

[1] Received July 31, 1973; revised Dec 11, 1973; accepted Jan 8, 1974.

[2] Vincristine: CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

[3] Medical Oncology, 2465 South Downing, Suite 206, Denver, Colo 80210.

[4] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

[5] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

TABLE 1.—Physical activity of patients in relation to onset of neuropathy

Group No.	No. of patients	Average No. of weeks to onset of neuropathy	Range (wks)
I	5	4.4	3–6
II, III	9	7.7	6–10
IV	6	10.3	7–14

Three of five patients in group I and five of six patients in group IV had T_4 levels recorded:

Group I, T_4: 2.8, 5.5, and 6.4 μg/100 ml

Group IV, T_4: 5.6, 8.1, 7.1, 9.6, and 9.4 μg/100 ml

The first patient in group I was taking 3 grains of thyroid extract as replacement while receiving chemotherapy. The three patients with elevated T_4 levels were diagnosed clinically as being euthyroid, as determined by the absence of nervousness, the absence of exophthalmos or other eye signs, the absence of tremor or increasing weakness, the absence of weight loss, and the absence of heat sensitivity.

Because of the limited number of patients in each group, statistical analysis of the above data was not performed. However, the data appear to indicate that the onset of peripheral neuropathy was directly related to the physical activity of each patient: 4.4 weeks (group I) versus 10.3 weeks (group IV) (table 1).

The extent and severity of disease in the patients of group I were compared with those of group IV in relation to estimated tumor volume and the obstruction of a major duct or passageway of a vital organ, eg, bronchus or bile duct. One patient in group I had obstruction of a major bronchus, and one patient in group IV had obstruction of the common bile duct. Two of five patients in group I and five of six patients in group IV had a total tumor mass estimated to be greater than 8 cc. Thus, the extent and severity of disease did not appear to be related to the onset of neuropathy.

DISCUSSION

Because the number of T_4s is so limited, no conclusion can be drawn concerning thyroid function and the onset of vincristine neuropathy except to suggest that an elevated T_4 may possibly play some role as a factor in the tolerance to vincristine (neuropathy).

Various factors which relate to the onset of vincristine neuropathy have been proposed, eg, total dose (1,2) or pre-existing neuropathy (6). Data are presented which suggest some correlation with T_4 levels. Also, the severity of the neoplastic disease in regard to its disabling features appeared to be involved, but this theory was discarded because no correlation could be found between the extent and severity of disease and vincristine neuropathy. Obviously, the extent and severity of disease can limit the activity of these patients. But more important than the degree of neoplastic involvement was the mental attitude of each patient to his disease. A few patients were so overwhelmed by the knowledge of having "cancer" that they engaged in only the most limited physical activity. These were the patients who appeared to manifest neurotoxicity the earliest. Conversely, those who were strenuously active appeared to tolerate vincristine without neuropathy for the longest periods of time. There were no significant differences between the incidence of objective versus subjective manifestations of neurotoxicity for those patients in groups I and IV. The use of Frenster's low-dose combination chemotherapy obviated the factor of interaction of other chemotherapeutic drugs (by maintaining them in fixed doses) and standardized the dose level of vincristine administered.

A review of the records of patients who are receiving various chemotherapeutic combinations which include vincristine in cancer centers where large numbers of patients are being treated could readily refute or affirm the observations reported in this paper. Also, the

role of elevated T_4 levels in the late appearance of clinical vincristine neuropathy appears worthy of further study.

CONCLUSION

We conclude that the onset of clinical vincristine neuropathy is a function not only of the total dose administered but also, possibly, a function of the level of physical activity.

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High-Dose Cyclophosphamide (NSC-26271) Therapy in Childhood Acute Leukemia [1,2,3]

C. Philip Steuber, Donald J. Fernbach, Kenneth A. Starling, and John M. Falletta [4,5]

Cyclophosphamide has been employed extensively as a major chemotherapeutic agent in the treatment of childhood neoplasms since its development in the late 1950s (1,2). More recently it has become commonly used in the preparation of bone marrow transplant recipients. Doses of 45–60 mg/kg/day given intravenously (iv) for 4 days were found to be tolerable even though severe toxicity occurred (3–5). Because of a paucity of information in the literature on high-dose cyclophosphamide treatment without subsequent bone marrow transplantation, a study was designed to evaluate the effectiveness of high-dose cyclophosphamide in inducing remission in children with advanced acute leukemia and to evaluate autologous marrow recovery after eradication of the leukemic blast population.

PATIENTS AND METHODS

Sixteen children with advanced acute leukemia in relapse were treated with cyclophosphamide at a dose of 40 mg/kg/day by rapid iv infusion (over 1 hour) for 4 days (*see* table 1). The average duration of illness prior to this treatment was 16.7 months. Six patients had hypocellular bone marrow patterns at the time treatment was begun. This was presumed to be secondary to earlier chemotherapy. Fourteen patients had previously received cyclophosphamide as an induction or maintenance agent or both. Six were receiving adrenal corticosteroids at the time the study was begun and the drugs were continued throughout. Symptomatic support was given as indicated.

Daily peripheral blood counts were obtained until remission occurred or continuing relapse was established. Bone marrow specimens were obtained before treatment (as shown in table 1) and, depending upon the clinical indication, at 1–4-week intervals after that. The differential criteria of the Southwest Oncology Group were used to evaluate the marrow specimens.

RESULTS

Five patients with acute lymphocytic leukemia (ALL) achieved bone marrow remission on Days 14, 16, 19, 24, and 35. All but patient No. 7 (who had not received cyclophosphamide previously) had severely hypocellular marrows.

In these responders, a rise in peripheral blood neutrophils occurred between 8 and 14 days after the completion of therapy. Platelet and erythroid recovery characteristically lagged 3–7 days behind myeloid recovery. All patients with ALL who were felt to have responded by our criteria were again in relapse (M_3) 4–6 weeks after recognition of M_1 or M_2 bone marrow status. Because of continuing depression of peripheral blood counts, patients Nos. 4 and 7 did not receive maintenance therapy. Two patients (Nos. 5 and 6) received an additional 2-day course of cyclophosphamide given iv on the 14th and 15th and on the

[1] Received Sept 28, 1973; revised Feb 20, 1974; accepted Mar 5, 1974.

[2] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate.

[3] Supported in part by Public Health Service training grant CA-08017 and research grants CA-03161 and CA-07357 from the NCI, National Institutes of Health, Department of Health, Education, and Welfare.

[4] Department of Pediatrics, Baylor College of Medicine, and the Research Hematology Laboratory, Texas Children's Hospital, Houston.

[5] We are grateful to Dr. Barbara J. Olivos and Dr. R. Ronald Jones for their assistance in this study.

TABLE 1.—Characteristics and responses of patients treated with high-dose cyclophosphamide

Patient No.	Age (yrs)	Duration of disease (mos)	Diagnosis	Previous cyclophosphamide	Marrow cellularity* (pretreatment)	Posttreatment marrow		Duration of remission† (wks)
						Days from start of study	Response/ cellularity*	
1	3	2	AML	+	D	19	M_1/D	12
2	14	21	AML	+	D	14	M_1/D	2
3	17	11	ALL	+	N	19	M_1/D	6
4	4½	16	ALL	+	N	14	M_1/D	4
5	6	9	ALL	+	N	16	M_1/D	6
6	8½	36	ALL	+	N	24	M_2/D	5
7	11	5	ALL	0	N	35	M_1/N	6
8	3½	17	ALL	+	D	43	M_2/I	—
9	4	13	ALL	+	D	21	M_2/D	—
10	4½	4	ALL	+	I	28	M_2/I	—
11	10	58	ALL	+	D	14	M_2/D	—
12	14½	6	ALL	0	I	32	M_2/I	—
13	3	34	ALL	+	D	Died, Day 9	—	—
14	4½	20	ALL	+	N	Died, Day 14	—	—
15	9½	19	ALL	+	N	Died, Day 18	—	—
16	8½	14	ALL	+	D	Died, Day 12	—	—

*D = decreased; N = normal; I = increased.
†Duration of remission from onset of M_1 or M_2.

24th and 25th days respectively. Patient No. 3 was treated biweekly with methotrexate[6] as a maintenance agent. Of the nine remaining patients with ALL, five showed marrow evidence of progressive disease between Days 14 and 43 and four died during the study period, three of infection and one of hemorrhage.

Both patients with acute myelogenous leukemia (AML) achieved hypocellular marrow remission. Patient No. 1 received maintenance therapy with cyclophosphamide given orally and died at 12 weeks with infection. A marrow specimen obtained 4 days before his death did not show active disease; however, postmortem examination revealed extensive visceral infiltration. Because of marrow depletion, no maintenance therapy was given to patient No. 2, who was in obvious relapse after only 2 weeks of "remission."

[6] Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)-methyl]methylamino]benzoyl]-.

TOXICITY

Anorexia, nausea, and/or vomiting of various degrees were universally observed but were controlled when necessary with antiemetics given iv without significant problem. Hematuria, which lasted 2–5 days, developed in four patients on the sixth, seventh, eighth, and 18th days of treatment. All patients received antibiotics during or subsequent to the period of cyclophosphamide administration. Pancytopenia developed in all of the children and eventually all patients received red blood cell or platelet transfusions or both. Those who had not previously lost their hair had total alopecia.

DISCUSSION

Sixteen children with advanced leukemia were treated with a modified high-dose cyclophosphamide regimen. Seven responded on the basis of marrow differential (M_1 and M_2) disregarding specimen cellularity. The average

duration of remission was 40 days. Resistance to low-dose cyclophosphamide had been recorded in 14 of these 16 patients. One of the two children who had not received prior cyclophosphamide therapy had a normocellular (M_1) marrow after treatment and an unmaintained remission of 6 weeks. A major problem at this advanced disease stage is the lack of any effective therapy for maintaining remission when it occurs.

The question arises whether a higher dose of cyclophosphamide would have elicited a response in those children who had progressive disease. In a similar patient population, Graw et al (6) gave cyclophosphamide, 45 mg/kg/day, for 4 days followed by transplantation. The nine patients who responded averaged 67 days in remission. Santos et al (7) reported an average survival time of 39 days following marrow transplantation after giving 50–60 mg/kg/day for 4 days. The major factor influencing survival in their series was graft-versus-host reaction rather than relapse. Animal and human studies have suggested that high total doses of cyclophosphamide (200–240 mg/kg) may cause drug-related deaths (8). However, in spite of severe toxicity, it is evident that at this dosage, remission can be induced and the bone marrow can regenerate.

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Role of a Nitrosourea (CCNU, NSC-79037) in Advanced Nonhematologic Cancer [1,2,3]

Marjorie Perloff, [4,5] Franco M. Muggia,[5] and Charles Ackerman [5,6]

Brief Reports and Preliminary Communications

CCNU is one of three nitrosourea derivatives currently undergoing clinical trials in patients with advanced cancer. These compounds have aroused interest because of their (*a*) dramatic effectiveness against certain animal tumors, notably murine L1210 leukemia and Lewis lung carcinoma (1); (*b*) ability to induce remissions in patients with malignant lymphomas resistant to conventional alkylating agents (2); (*c*) uniquely delayed hematologic depression [7]; and (*d*) high lipid solubility and ability to cross the blood-brain barrier (3). The mechanism of action of CCNU has not been completely delineated but it functions as an alkylating agent, as an inhibitor of enzymatic processes leading to the formation of DNA, and as a modifier of cellular proteins (4). Although it is cell cycle nonspecific and kills both proliferating and nonproliferating cells, CCNU has also been shown to double the duration of the mitotic S phase in L1210 ascites leukemia (5).

In the initial clinical trials a dose of 130 mg/m^2 given orally every 6 weeks proved optimal (6). The only consistent dose-limiting toxic effects were delayed hematologic toxic reactions with a median nadir white blood cell (wbc) count on Day 42 and a median nadir platelet count on Day 28.

The present study was undertaken to determine the spectrum of activity of CCNU in a group of patients with advanced malignancies that are relatively unresponsive to other chemotherapeutic agents. Previous reports have established the usefulness of the nitrosoureas in the treatment of patients with malignant gliomas (7), multiple myeloma (1), and malignant lymphomas, particularly of the Hodgkin's type (2).

MATERIALS AND METHODS

Fifty patients with histologically documented malignancies were evaluated. All patients had a wbc count greater than 5000 cells/mm^3, a platelet count greater than 100,000 platelets/mm^3, a blood urea nitrogen level less than 25 mg/100 ml, and a bilirubin level less than 2 mg/100 ml.

Most patients had received prior therapy and subsequently showed progression of their disease. Among the 27 patients with lung carcinoma (ten epidermoid, five adenocarcinoma, six small cell, and six undifferentiated) 14 patients had received radiotherapy for their pulmonary lesions and seven of these patients had also received chemotherapy. Five other patients had been treated with extrapulmonary radiotherapy and eight other patients had received prior chemotherapy alone. Ten of the 13 patients with gastrointestinal adenocar-

[1] Received Oct 9, 1973; revised Dec 20, 1973; accepted Jan 24, 1974.

[2] Supported in part by Public Health Service grant CA-13330-01 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare; and The Chemotherapy Foundation of New York.

[3] CCNU: CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-. Supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

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[6] *Reprint requests to:* Franco M. Muggia, MD, Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461.

[7] Broder LE, and Carter SK. Clinical brochure: 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) NSC-79037. Prepared by the NCI, Bethesda, Md, 1971.

cinomas had been treated with 5-fluorouracil.[5] Six of the ten patients with other tumors (sarcoma, melanoma, hypernephroma, thymoma, bladder) had received radiotherapy and two of these patients had also received some form of chemotherapy.

CCNU was administered as a single oral dose of 130 mg/m^2 along with oral phenothiazine drugs given as antiemetics. Following the first course of therapy, complete blood counts were obtained at 1-, 2-, or 3-week intervals. Patients were reevaluated after 6 weeks. If the nadir wbc and platelet counts during the first course of therapy were not lower than 2000 cells/mm^3 and 50,000 platelets/mm^3 respectively, patients were then eligible to receive the initial dosage at 4-week intervals.

RESULTS

Toxic Reactions and Complications

During the first course of therapy, nausea and vomiting that occurred in 26 of 50 patients were mild to moderate and were not responsible for significant morbidity.

Hematologic toxicity was similar to that previously reported (6). Thrombocytopenia (a platelet count less than 100,000 platelets/mm^3) occurred in 18 patients. Clinically apparent gastrointestinal bleeding was present in five of these patients. Six significant episodes of infections occurred in the 12 patients who became leukopenic (wbc count less than 3000 cells/mm^3) during treatment; five of these patients had lung cancer. Recovery from hematologic toxic effects was also similar to that previously reported (6). Nadir platelet counts were present at Day 28 and recovery was apparent within 10 days. Normalization of wbc counts occurred within 10 days following a nadir at 6 weeks.

Fourteen patients received two courses of CCNU, three patients received three courses, and two patients received six courses. Each of these courses was given at 4-week intervals. Although subsequent hematologic data were not always collected at 2-week intervals, there was evidence of cumulative hematologic toxic effects in only one patient.

Therapeutic Responses

Partial responses (a greater than 50% decrease in measurable lesions) were seen in six of 27 patients with lung cancer (one epidermoid, one adenocarcinoma, two small cell, and two undifferentiated) and one of three patients with malignant melanoma. The median duration of these responses was 3 months. The longest responses were obtained in the patient with melanoma (subcutaneous nodule and lymph nodes) (13+ months) and in the patient with lung adenocarcinoma (12+ months). The only complete response was in a patient with a biopsy-documented recurrent liposarcoma that was of 3 months' duration. There were no objective responses in the 13 patients with gastrointestinal adenocarcinoma, but there were three subjective responses.

Among the five patients who had brain metastases at the time of CCNU treatment, two patients with lung cancer showed neurologic improvement for 1 and 4 months respectively. One other patient with lung cancer and two patients with colon cancer who had cerebral metastases showed no response to the treatment.

DISCUSSION

The current study describes our experience with CCNU in the treatment of 40 patients with carcinomas of pulmonary and gastrointestinal origin and in the treatment of ten patients with a variety of malignancies. A dose of 130 mg/m^2 was found optimal only in patients who had no prior extensive treatment with radiotherapy. The current experience suggests that in order to avoid marked thrombocytopenia and its complications in patients who have received extensive radiotherapy, these patients should receive an initial dose of CCNU of 100 mg/m^2 as has been recently reported (8). Not enough experience was accrued on an every-4-week schedule to demonstrate the safety of this particular shorter schedule; however, it appears feasible to re-

[5] 5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU.

peat this dose at 4-week intervals if there is clinical urgency and if the platelet count has begun to rise. A recent study confirms this suggestion (9).

The infrequent therapeutic responses observed in this study must be viewed in perspective, considering the generally low response of such tumors to chemotherapy, particularly if other therapeutic modalities have been previously employed. In bronchogenic carcinoma CCNU has been found useful occasionally, although the high response rate previously reported in the epidermoid cell type (10) was not confirmed by us. A recent study by the Veterans' Administration Lung Cancer Study Group indicated that the related nitrosourea, BCNU,[9] was not superior to cyclophosphamide [10] in any cell type except perhaps in large cell undifferentiated carcinoma (11). However, this study indicated a larger number of long-term survivors among lung cancer patients treated with BCNU rather than with cyclophosphamide.

Some patients with advanced gastrointestinal adenocarcinoma relapsing after 5-fluorouracil treatment benefited subjectively from CCNU treatment. The tolerable treatment program renders CCNU worthy of trial in this group of patients. Although the percentage of responses observed following CCNU therapy is inferior to those responses following 5-fluorouracil therapy, CCNU has shown modest activity against such carcinomas (12).

The nitrosoureas have shown definite therapeutic activity in malignant melanoma (13); one of three patients treated in our study obtained a remission. Occasional tumor regression is also seen in patients with renal cell carcinoma (14) and sarcoma (15). The only complete response in the present series was obtained in one such patient with metastatic liposarcoma. The possibility that the unique lipid solubility of these agents renders this particular sarcoma especially susceptible to the nitrosoureas requires further study. Although we had no experience in the current study, patients with carcinoma of the breast rarely benefit from treatment with nitrosoureas (16,17).

In conclusion, CCNU is one of three nitrosourea compounds presently undergoing clinical trials. Its toxic manifestations have been quite well defined and its spectrum of activity is becoming increasingly well delineated. In order to enhance its usefulness, CCNU should have a more frequent schedule of administration and be used in combination with other agents; in addition, predictive methods to determine tumor responsiveness need further exploration.

[9] BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-.

[10] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)-amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

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ANNOUNCEMENTS

First Postgraduate Course on Medical Oncology

Milan, June 17–21, 1974
Associazione Italiana di Oncologia Medica (AIOM)
Istituto Nazionale per lo Studio e la Cura dei Tumori di Milano
Lega Italiana per la Lotta Contro i Tumori, Sezione Milanese

Chairman: Umberto Veronesi, MD
Program co-ordinator: Gianni Bonadonna, MD
Secretary: Silvio Monfardini, MD

The subscription fee for this postgraduate course is 50.000 Italian lirè. This includes the lunch and the syllabus. All lectures will be delivered in Italian. For information and subscription, contact: Miss Lucia Manfredi, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via Venezian 1, 20133 Milan, Italy (tel. 2390, ext. 214).

Monday, June 17th

Morning

Introduction: The medical oncology and the neoplastic patient. (G. Bonadonna)
Principles of cell kinetics of neoplastic cells. (R. Silvestrini)
Principles of pathophysiology of human tumors. (M. Fiorentino)
Immune reaction in tumors. (G. Della Porta)

Afternoon

Mechanism of action of anticancer drugs. (R. Rosso)
Side effects of anticancer drugs. (M. Fiorentino)
Panel Discussion: Clinical pharmacology of anticancer drugs.
Chairman: M. Ghione
Discussants: G. Di Fronzo; G. Lenaz; P. Periti; R. Rosso; R. Silvestrini

Tuesday, June 18th

Morning

Modern aspects of pathology integrated into clinical oncology. (F. Rilke)
Immunologic tests in clinical practice. (S. Del Giacco)
Mode of spread of tumors. (M. Fiorentino)
Staging classification of tumors. (R. Molinari)

Afternoon

Panel Discussion: Clinical methods for evaluation of an anticancer drug.
Chairman: G. Bonadonna
Discussants: F. Calabresi; M. Fiorentino; S. Monfardini; A. Morabito; R. Rosso
Principles of surgery in the treatment of cancer. (B. Salvadori)
Principles of radiotherapy in the treatment of cancer. (U. Felci)

Wednesday, June 19th

Morning

Single agent chemotherapy and combination chemotherapy. (G. Bonadonna)
Immunotherapy of tumors. (G. Luporini)
Adjuvant chemotherapy to surgery and radiotherapy. (M. De Lena)
Natural history and diagnostic evaluation of Hodgkin's disease. (A. Barduagni)

Afternoon

Natural history and diagnostic evaluation of non-Hodgkin's lymphomas. (M. Pergola)
Staging laparotomy in malignant lymphomas. (G. Bonadonna)
Panel Discussion: Therapeutic strategy in the management of malignant lymphomas as related to the extent of disease.
Chairman: A. Banfi
Discussants: M. De Lena; M. Fiorentino; S. Tura; R. Zucali

Thursday, June 20th

Morning

Clinical evaluation and medical treatment of bronchogenic carcinomas. (G. Tancini)
Clinical evaluation and chemotherapy of gynecologic tumors. (G. De Palo)
Clinical evaluation and chemotherapy of testicular tumors. (S. Monfardini)
Chemotherapy of soft tissue sarcomas and malignant melanoma. (G. Beretta)

Afternoon

Principles of diagnosis and treatment of chronic leukemias. (G. L. Castoldi)
Natural history and treatment of multiple myeloma. (G. Luporini)
Panel Discussion: Therapeutic strategy and results in the management of acute leukemias.
Chairman: S. Monfardini
Discussants: G. L. Castoldi; F. De Cataldo; M. Fiorentino; E. Madon; A. Marolla; L. Massimo

Friday, June 21st

Morning

Endocrine treatment of malignant tumors. (S. Di Pietro)
Combination chemotherapy in the various stages of breast cancer. (M. De Lena)
Chemotherapy of miscellaneous solid tumors (gastrointestinal tract, head and neck, bone, urinary tract, etc). (M. Pergola)
Diagnosis and treatment of infections in patients with cancer. (E. Bajetta)
Metabolic complications in neoplastic disease and their management. (M. Fiorentino)

Afternoon

The management of ambulatory patients in medical oncology. (S. Monfardini)
General aspects of pediatric tumors. (F. Fossati)
Panel Discussion: Therapeutic strategy and results in pediatric tumors.
Chairman: E. Madon
Discussants: L. Di Gagno; F. Fossati; M. Gasparini; E. Grigoletto; L. Massimo; R. Zucali

Fellowship in Medical Oncology

Fellowships are available in Medical Oncology at the City of Hope National Medical Center, Duarte, California, for one or two years. The program involves participation in a Cooperative Drug Evaluation Program (Western Cancer Study Group). In addition, opportunities will be provided to participate in the activities of the Hematology, Radiation Therapy, and Pathology Services. Two years of Medical Residency and California Licensure are required. The beginning stipend is $17,000.

Inquiries should be directed to Dr. Joel Solomon, Senior Physician, Hematology Department, City of Hope National Medical Center, 1500 E Duarte Road, Duarte, Calif 91010.

Postgraduate Course on the Diagnosis and Treatment of Lung Cancer

From June 3 to 7, 1974, the National Cancer Institute of Milan and the Italian League Against Cancer, Milan Section, will organize a Postgraduate Course on the Diagnosis and Treatment of Lung Cancer. (Chairman, Prof. U. Veronesi; co-ordinator, Prof. G. Ravasi; secretary, Dr. M. De Lena.)

Subjects of the lectures will be: epidemiology, etiology, natural history, histopathology, diagnostics, classification, radiotherapy, and chemotherapy. Experimental as well as clinical subjects will be treated.

A large group of Italian experts will be present as well as the following foreign lecturers: Klastersky (Belgium); Higginson (Israel); Tubiana (France); Deeley, Gough, and Paneth (Great Britain); Brunner (Switzerland); Mountain, Saffiotti, and Selawry (USA).

Four panel discussions will be held on the following subjects: "The primary prevention of lung cancer," "Possibilities and limits of preclinical diagnosis of lung cancer," "Results and prognosis of surgical treatment in lung tumors," "Controlled clinical trials: outlook for treatment of lung cancer," and also closed circuit TV presentations of surgical operations and diagnostic procedures.

Simultaneous translation will be provided in English, French, and Italian.

The subscription fee is Lit. 60,000 (including the proceedings of the course, lunch, etc). Because the number of participants is limited, please send subscriptions as soon as possible to: Miss Lucia Manfredi, Ufficio Attività Didattiche, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via G. Venezian, 1, 20133 Milan, Italy (tel 2390 ext 214).

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U.S. Department of Health, Education, and Welfare
Public Health Service **National Institutes of Health**
DHEW Publication No. (NIH) 74-22